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### ATR is Required for a Mitotic Entry Checkpoint that Prevents Premature Chromatin Condensation after UV Exposure

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ATR (Ataxia Telangiectasia and Rad3-related) is homologous to yeast protein kinases required for survival after UV. In a separate abstract we describe the generation of stable human cell lines inducible for wild type or a dominant negative (kinase dead) isoform of ATR. Expression of the kinase dead isoform (ATR-kDa) markedly sensitized cells to multiple types of DNA damage but did not affect p53 function or G1 arrest after damage. Without damage, ATR-kDa had no effect even when expressed for two weeks. In the presence of low dose damage (UV, ionizing radiation or hydroxyurea), ATR-kDa expressing cells underwent profound chromosomal fragmentation as revealed by mitotic spreads. This fragmentation was analogous to that described as "premature chromatin condensation" (PCC) induced by caffeine and DNA damage (Schlegel & Pardee, Science, 1986). Caffeine has recently been shown to inhibit ATR protein kinase activity *in vitro*. Indeed, we find *in vivo* that ATR-kDa promoted caffeine's ability to sensitize to DNA damage, while ATR-wt rescued cells from this radiosensitizing effect of caffeine. Cell synchronization experiments showed that ATR is most important in mid-S phase to prevent PCC and apoptosis. Consistent with a presumed role downstream of ATR, adenoviral expression of the kinases Chk-1 or Chk-2 rescued PCC caused by ATR-kDa, while expression of the dominant negative isoforms of these kinases promoted PCC. Because they often lack a DNA damage-responsive G1 checkpoint, cancer cells are often more sensitive to the loss of later cell cycle checkpoints than normal cells. We mimicked the loss of the G1 checkpoint by adenoviral expression of G1/S cyclins (cyclin E or CDK2). Indeed, expression of these cyclins synergized with ATR inhibition to cause increased sensitivity to damage. This result coupled with the sensitivity of S phase cells to ATR inhibition suggests ATR as a possible target for a radiosensitizing agent. We conclude that ATR plays an essential role in a checkpoint which prevents lethal premature mitosis in cells which have received low dose DNA damage.

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### Differences in Proliferation and Maturation Between Normal and Mastocytosis Mast Cells

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We have examined the development of mast cells (MCs) in normal skin and mastocytosis lesions by analyzing the expression of proliferative cell nuclear antigen (PCNA) and the BCL-2 oncoprotein with specific mAbs. MCs were identified with conjugated avidin and mAbs for chymase and tryptase. In normal skin, less than 2% of MCs expressed PCNA and all cells were BCL-2-negative, whereas 30–95% of mastocytosis lesional skin MCs expressed PCNA and 10–45% of these MCs were BCL-2 positive. To confirm these observations *in vitro*, normal skin MCs in culture and the mastocytosis cell lines (HMC-1 and RMT) with activating c-kit mutations were also examined. Normal skin MCs cultured in medium alone had less than 2% PCNA-expression and all of these cells were BCL-2-negative. With the addition of stem cell factor (SCF, 30 ng per ml) to these cells for 7 days, 25% of normal MCs expressed PCNA, but none dividing MCs were observed and the total number of MCs decreased by 8% when compared to the added number of cells at day 0. All these cells remained BCL-2-negative. In contrast, more than 95% of the mastocytosis cell lines expressed both PCNA and BCL-2. The results of this study indicate that mastocytosis MCs with c-kit auto-activation in tumor and culture are highly proliferative and these cells may avoid apoptosis by expressing BCL-2 oncoprotein. In contrast, normal skin MCs in tissue and culture appear to have very low proliferation activity and do not express BCL-2. These latter observations have led us to propose that SCF activation of c-kit in normal skin MCs leads primary to maturation rather than proliferation.

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### Up-Regulated L-Selectin and Diminished Integrin Expression in Sézary Cells: Functional Consequences Under Physiologic Shear Stress Conditions

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Although aberrations in adhesion molecule expression by lymphoma cells have been reported, the functional consequences of these changes are unclear. Herein, we report a patient with Sézary syndrome whose peripheral blood T cells (90%) consisted of a TCRVβ17<sup>+</sup> malignant clone as detected by TCRVβ-specific mAb. Thin plaques which showed a dense infiltrate of TCRVβ17<sup>+</sup> cells were present on 70% of the patient's body surface. By flow cytometry, peripheral blood malignant T cell adhesion molecule abnormalities included an 80% down-regulation of LFA-1 compared to normal controls and no detectable expression of α4 integrin. Under shear stress conditions (1.5 dyne per cm<sup>2</sup>) in a parallel plate flow chamber, malignant T cells failed to arrest on recombinant ICAM-1 in the presence of several chemokines and displayed an 80% decrease in the ability to arrest on TNF-α activated dermal microvascular endothelial cells compared to normal CD4<sup>+</sup> memory T cells (mTC). Cutaneous lymphocyte-associated antigen (CLA) expression was detected in ~25% of malignant T cells in the peripheral blood, but was expressed in <10% of TCRVβ17<sup>+</sup> T cells in the dermis. By contrast, 95% of malignant T cells in peripheral blood expressed L-selectin (CD62L), and (as others have reported with CTCL) L-selectin ligand was detected in normal blood vessels at affected skin sites by MECA79 mAb. Compared to normal CD4<sup>+</sup> mTC, malignant T cells expressed 2-fold more L-selectin and rolled in 6-fold greater numbers/unit area on L-selectin ligand (n = 5, p < 0.0001). Thus, aberrant expression of integrins led to striking defects in Sézary cell arrest on activated endothelial cells *in vitro*. Malignant T cells in this patient, however, entered skin and produced significant clinical disease. We propose that up-regulated expression of L-selectin may be a novel mechanism by which malignant T cells may target skin in greater numbers and compensate for diminished integrin expression.

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### The C-Kit Signaling Pathways in Normal and Mastocytosis Mast Cells

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The C-kit receptor is a tyrosine kinase vital in the development and survival of mast cells (MCs). Mutated c-kit autoactivation is associated with the development of adult mastocytosis. Mitogen activated kinases (MEKs) and Janus kinases (JAKs) are pivotal enzymes in c-kit signaling pathways. We examined the roles of JAK-3 and MEKs on the proliferation of mastocytosis cell lines HMC-1 and RMT, which express autoactivated c-kit. When added to these cells for 3 days, the JAK-3 inhibitor WHI-P131 (5 and 20 μM) decreased the number of HMC-1 by 53 ± 14% and 61 ± 19% and RMT by 46 ± 8% and 58% ± 19%, when compared to controls. The MEKs inhibitor UO126 (5 and 20 μM) also reduced the growth of HMC-1 (47 ± 7% and 54 ± 11%) and RMT (23 ± 9% and 33 ± 10%). When stem cell factor (SCF, 30 ng per ml) was added to these cultures, it did not significantly reverse the growth inhibiting effects of both WHI-P131 and UO126. Neither inhibitor induced significant cell death in these cultures. We also investigated the role of c-kit on normal skin MCs using *in vitro* MC maturation model. When added to neonatal foreskin cell cultures that containing MC precursors for 7 days, SCF (30 ng per ml) enhanced the number of mature MCs to 34 ± 15% above controls. The JAK-3 inhibitor did not alter this SCF maturation effect. These results demonstrate that JAK-3 and MEKs are important enzymes in the signaling pathways of auto-activated c-kit. Since their inhibition results in decreased mastocytosis MC proliferation, the primary role of these kinases appears to be in cell division. In contrast, JAK-3 appears play little role in normal skin MC maturation because inhibition of the enzyme did not reduce the number of mature MCs in culture. We conclude that c-kit play an important role in MC proliferation and maturation, but the c-kit signaling pathways for MC proliferation and maturation differ.

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### Folliculotropic Mycosis Fungoides with Central Nervous System Involvement

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Folliculotropic mycosis fungoides is a rare variant of cutaneous T-cell lymphoma, mycosis fungoides (MF) type characterized by atypical lymphocytes preferentially infiltrating the hair follicle epithelium relative to the epidermis. We describe the first case in which laser capture microdissection was used to show that the atypical lymphocytes within the hair follicle epithelium were part of the same tumor clone as those in the epidermis and the patient's blood. This is also the first case of folliculotropic mycosis fungoides with involvement of the central nervous system. The successful procurement and analysis of atypical lymphocytes from hair follicle epithelium by laser capture microscopy ushers in a new era in molecular diagnostics. In reviewing the literature describing atypical lymphocytes infiltrating hair follicle epithelium relative to the epidermis, we encourage the use of the term "folliculotropic MF." Our case also supports previous findings in classic MF patients that CNS involvement can occur in advanced disease.

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### Borrelia Burgdorferi is Absent in Cutaneous B-Cell Lymphomas within the United States

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An association between Borrelia burgdorferi and cutaneous B-cell lymphoma (CBCL) has been made in several European countries. The evidence in favor of such an association has recently been based on more definitive tests for the pathogenetic role of Borrelia burgdorferi in CBCL, including positive cultures or PCR amplification of borrelial DNA from lesional skin. However, there has been only one negative report of Borrelia burgdorferi in five North American cases of CBCL. Therefore, we retrieved 38 cases of primary and secondary CBCL from different geographic locations of the United States. Two separate techniques were used to detect borrelial DNA by PCR, a nested PCR method to amplify a Borrelia burgdorferi-specific gene as well as a borrelial chromosomal Ly-1 clone amplification method. Southern blot hybridization was used for confirmation of the PCR results. No Borrelia burgdorferi-specific DNA was detected in any of the 38 CBCL cases, whereas detectable PCR products were obtained with our positive controls. Our findings suggest that Borrelia burgdorferi plays no role in the development or pathogenesis of CBCL in the United States. The findings also suggest that the geographic variation of the clinical manifestations of Borrelia burgdorferi is indeed real and may be secondary to the genetic and phenotypic differences between species present in Europe and North America.

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**Fas/Fas Ligand-Mediated Elimination of Cytotoxic CD8+ Lymphocyte in Mycosis Fungoides: A Potential Mechanism of Tumor Immune Escape?**

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Mycosis fungoides (MF) is an indolent cutaneous T-cell lymphoma (CTCL) of skin homing, CD4+/CD45RO+ (helper/memory) T-lymphocytes. Loss of CD8+ cytotoxic T cells in MF lesions is associated with poor prognosis. T-cell apoptosis controlled by Fas/Fas ligand (FasL) system allows FasL+ tumors escape immune surveillance, triggering apoptosis of tumor infiltrating T-lymphocytes. To evaluate the potential role of Fas/FasL-mediated apoptosis, immunohistochemical staining and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) were used to study FasL expression and apoptosis in MF lesions and paired, uninvolved skin specimens taken from 21 patients. Western Blotting showed FasL expression in CTCL cell lines (MJ, Hut78 and HH). FasL staining was positive (1–3+) in epidermal keratinocytes (17/21) and infiltrating lymphocytes (13/21) in MF lesions and higher in stage IV (5/6). FasL staining was more intense in MF dermal infiltrates than in paired, uninvolved MF skin (10/15, sign test,  $p < 0.01$ ). Strongest FasL expression was seen colocalizing with CD45RO+ cells with cerebriform nuclei. The number of CD8+ cytotoxic T lymphocytes in FasL+ areas ( $2.01 \pm 0.86\%$ ) was significantly less than in FasL– areas ( $13.53 \pm 3.54\%$ , Wilcoxon signed ranks test,  $p < 0.02$ ). Increased TUNEL-positive epidermal keratinocytes (13/15) and dermal lymphocytes (13/15) were present in MF lesions and paired uninvolved skin. By CD8/TUNEL doublestaining, the majority of CD8+ cytotoxic T lymphocytes were TUNEL-positive. These data suggest that FasL expression by MF tumor cells may lead to apoptosis of CD8+ cytotoxic T lymphocytes by the Fas/FasL pathway. Fas/FasL mediated elimination of CD8+ cytotoxic T lymphocytes may thus be a potential mechanism of tumor immune escape in mycosis fungoides.

## 603

**Cutaneous Lymphomatoid Granulomatosis: A Clinical, Microscopic and Molecular Study**

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Lymphomatoid granulomatosis (LYG) is a rare and unique angiocentric and angiodestructive Epstein-Barr virus associated B-cell lymphoproliferative disorder (EBV-BLPD), varying widely from an indolent process to an aggressive large cell lymphoma. The skin is the extrapulmonary organ most commonly involved in LYG. We studied 31 skin lesions from 20 patients with known pulmonary LYG, using immunohistochemistry, *in situ* hybridization for Epstein-Barr virus (EBV), and PCR for the presence of antigen receptor gene rearrangements (IgH and TCR), to better define both the clinicopathologic spectrum and pathogenesis of the cutaneous lesions. We identified 2 distinct patterns of cutaneous involvement. Multiple erythematous dermal papules and/or subcutaneous nodules, with or without ulceration, were present in 17 patients (85%). These lesions demonstrate a marked angiocentric lymphohistiocytic infiltrate, composed predominantly of CD4-positive T-cells, with a high propensity for involving the subcutaneous tissues, and exhibiting angiodestruction, necrosis, and cytologic atypia. EBV-positive B-cells were detected in the nodules from 5 patients; clonal immunoglobulin heavy chain gene (IgH) rearrangements were detected by PCR in 2 patients. Multiple indurated, erythematous to white plaques were present in 3 patients (15%). The plaque lesions uniformly lacked EBV and clonal IgH gene rearrangements. The clinical course of overall disease was variable, ranging from spontaneous regression without treatment (1/13; 7%), resolution with chemo/immunomodulatory therapy (8/13; 62%), and progression (4/13; 31%). The clinical and histopathologic features of cutaneous LYG are extremely diverse. However, the majority (85%) of the cutaneous lesions mirrors to some extent LYG in the lung, although EBV+ cells are less frequently identified. This subset of cases shows the histopathologic triad of angiodestruction with associated necrosis, panniculitis, and in some cases atypical lymphoid cells. A small percentage of the lesions (15%) contain only sparse lymphoid cells, are EBV-negative, and presented as indurated and atrophic plaques. The pathophysiologic basis of these two types of lesions appears to differ. While some cases of LYG regress spontaneously, most require therapy.

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**Expression and Distribution Patterns for the ErbB2 Receptor During Wound Repair**

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Although 4 isoforms of the EGF receptor family have been identified in human cells, the purpose of our study was to characterize the possible involvement of the receptor member known as ErbB2 (neu or HER-2) during wound repair. While this orphan receptor binds no known ligand, its expression has proven to be a poor prognostic indicator for many tumor types. To investigate the potential role of the ErbB2 receptor during wound healing, we selected a porcine wound model since we ultimately planned in future studies to modulate receptor expression using *in vivo* porcine studies. Since porcine sequences for EGF receptor isoforms were unknown, we began by cloning the porcine ErbB2 out of a cDNA library constructed from a day 7 partial-thickness excisional wound. At present we have sequenced the majority of the porcine ErbB2 gene. Expression of porcine ErbB2 was verified by Northern blotting using excisional wounds collected at days 4 and 7 after excisional injury. In our next experimental series, a specific ErbB2 antisera was used in immunohistochemical staining studies to evaluate the temporal and spatial distribution of this EGF receptor isoform. Porcine excisional injuries were examined first. Human burn wounds ( $N = 15$ ) were also examined to confirm that ErbB2 is relevant to the human wound environment. In nonwounded skin controls, ErbB2 protein was identified throughout the normal epidermal layers with a prominent plasma membrane distribution in the stratum granulosum. In wound margins or upwardly growing dermal sweat ducts or hair follicles, weak, patchy or absent ErbB2 immunoreactivity was observed whereas more differentiated keratinocytes in the stratum spinosum or granulosum, inner root sheaths or secretory segments of sweat glands showed increasingly stronger accumulations of immunoreactive ErbB2 protein. The ErbB2 receptor also appears to accumulate in endothelial cells lining all segments of the vasculature and is present in select dermal populations with a fibroblastic phenotype. While our earlier studies have shown that the ErbB1 receptor form plays a prominent role in wound healing, the present studies suggest that the ErbB2 receptor form is likewise up-regulated during normal wound healing. Our experiments show that ErbB2 expression is not exclusively associated with neoplastic proliferation and is not a constitutively expressed gene in keratinocytes. These data raise the possibility that the ErbB2 receptor form is available to form heterodimers with other ErbB receptor isoforms in at least 3 of the cell types that are critical to wound repair.

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**Three Pediatric Cases of CD30+ Cutaneous Anaplastic Large Cell Lymphomas (ALCLs) Bearing the t(2;5)(p23;q35) Translocation**

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The t(2;5)(p23;q35) translocation may be more strongly associated with younger patient age, rather than nodal vs. cutaneous site of presentation. Review of the available literature, in conjunction with our analysis of three pediatric cases of cutaneous CD30+ ALCLs with the translocation, support this conclusion. Our three cases of pediatric cutaneous ALCL all contained the t(2;5)(p23;q35) translocation as determined by PCR analysis of genomic DNA. Each case exhibited a unique breakpoint as evidenced by different sizes of the PCR products. All three cases had an excellent outcome. Two were stage IEA. One was stage IIIEA. Since its discovery in CD30+ ALCLs the t(2;5)(p23;q35) translocation has shown a high degree of association with nodal disease, younger patient age and a better prognosis. The translocation results in the fusion of the NPM nucleolar phosphoprotein housekeeping gene on chromosome 5q35 to the protein tyrosine kinase gene, anaplastic lymphoma kinase, on chromosome 2p23. As a result, anaplastic lymphoma kinase which is normally silent, is constitutively expressed. Most prior studies of the t(2;5) translocation in primary cutaneous ALCLs were restricted almost exclusively to adults. Therefore it is not surprising that the t(2;5) translocation has been rarely associated with pediatric cutaneous presentations. We conclude that the t(2;5) translocation is associated with younger patient age and better survival, but is not a marker for nodal disease.

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**Loss of PTEN Tumor Suppressor Gene Expression in the Pathogenesis of Trichilemmomas**

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Cowden syndrome (CS) is a genetic disorder characterized by multiple hamartomas and a higher risk of benign and malignant neoplasms of the thyroid, breast, endometrium, and skin. The most frequently occurring skin lesion in patients with CS is the trichilemmoma, a benign tumor with follicular infundibular differentiation. Patients with CS have recently been shown to have germline mutations of the *PTEN* tumor suppressor gene, which encodes a dual-specificity phosphatase at 10q23.3. We hypothesized that both CS and non-CS (sporadic) trichilemmomas may have a high frequency of *PTEN* mutation and subsequent loss of expression. Using immunohistochemistry, we examined *PTEN* expression in 16 trichilemmomas from 11 patients (2 CS, 14 sporadic). In all specimens, there was strong *PTEN* staining of melanocytes, Langerhans cells, and endothelial cells. Weaker staining was observed in the inner root sheath, outer root sheath, and infundibulum of the hair follicle, as well as in the keratinocytes and eccrine glands. In *PTEN* positive cells, staining was seen in both the cytoplasm and nucleus (or nuclear membrane). Both of the trichilemmomas associated with CS (from 2 patients) showed loss of *PTEN* expression. Eight of 14 non-CS, sporadic trichilemmomas (from 5 patients) also showed loss of *PTEN* expression. Of the 10 *PTEN* negative trichilemmomas, the loss of cellular *PTEN* expression was uniform in 8 tumors. In the remaining 2 trichilemmomas (from the same non-CS patient), both *PTEN* positive and *PTEN* negative cells were observed (heterogeneous expression). Loss of *PTEN* expression is seen in both CS-associated and sporadic trichilemmomas, which may imply a similar step in their pathogenesis. However, the fact that not all sporadic trichilemmomas were *PTEN* negative may suggest that loss of *PTEN* expression is associated with, but not causative of, some trichilemmomas. Alternatively, loss of *PTEN* expression may be an early, but not necessary, event that predisposes neoplastic transformation through other genetic pathways. The uniform loss of *PTEN* expression in 8 of the 10 *PTEN* negative tumors studied implies that this loss is an early event, which is propagated among all neoplastic cells. However, occasional tumors with heterogeneous *PTEN* expression are consistent with mutation occurring after initial neoplastic transformation.

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**Distinct Roles of Extracellular Signal-Regulated Kinase, Jun N-Terminal Kinase, and p38 Signaling Pathways in Enhancement of Matrix Metalloproteinase-1, -3, and -19 Expression in Dermal Fibroblasts**

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Matrix metalloproteinase-19 (MMP-19) is a novel MMP previously found in endothelial and synovial cells. We have examined the roles of mitogen-activated protein kinase (MAPK) signaling pathways in the regulation of MMP-19 expression in normal human skin fibroblasts. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) activates extracellular signal-regulated kinase (ERK)1,2, Jun – N-terminal kinase (JNK) and p38 MAPK in dermal fibroblasts. TNF- $\alpha$ -elicited induction of MMP-19 mRNA (4.7-fold) was inhibited in part (by 46%) by MEK1,2 inhibitor PD98059, that blocks the ERK1,2 pathway (Raf/MEK1,2/ERK1,2), and by a specific p38 MAPK inhibitor SB203580 (by 49%). In contrast, induction of collagenase-1 (MMP-1) and stromelysin-1 (MMP-3) mRNA levels by TNF- $\alpha$  was potentially reduced by SB203580 (by 76 and 90%, respectively), but not by PD98059. Infection of the cells with recombinant adenovirus for constitutively active MEK1 resulted in activation of ERK1,2, and markedly enhanced the mRNA levels and production of MMP-19, MMP-1, and MMP-3. Infecting cells with an adenovirus for constitutively active MKK3b or MKK6b (p38 kinase) specifically activated p38 MAPK and slightly enhanced the production of MMP-19. Co-infection of cells with constitutively active MEK1 in combination with active mutants of MKK7 (JNK kinase), MKK3b, or MKK6b potentially enhanced the MMP-19, MMP-1, and MMP-3 expression as compared to infection with constitutively active MEK1, MKK3b, or MKK6b alone. The activation of ERK1,2 resulted in induction of *c-jun*, *jimB*, and *c-fos* expression, whereas activation of p38 alone had no effect on their expression. These results identify two distinct mechanisms for inducing MMP-1, MMP-3, and MMP-19 expression in fibroblasts: AP-1-dependent activation via ERK1,2 pathway, and AP-1-independent activation via p38 pathway, both of which apparently control the proteolytic activity of normal fibroblasts, e.g. in wound repair and tumor invasion.

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### Interstitial Collagenase (MMP-1) is Induced in Dermal Fibroblasts through Interleukin-1 Secreted by Epidermal Keratinocytes

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When human skin is maintained in organ culture under growth factor-free conditions, normal architecture is preserved for several days. When exogenous growth factors such as epidermal growth factors (EGF) or hepatocyte growth factor (HGF) are included in the culture medium, invasion of the underlying stroma by epithelial cells occurs. Invasion is dependent on MMP-1 (interstitial collagenase) elaboration by dermal fibroblasts. In the present study, we have investigated how MMP-1 production is regulated during invasion. Human foreskin fibroblasts were exposed to culture fluid from epidermal keratinocytes. After 48 h incubation, fibroblast culture fluids were assessed for MMP-1 using casein zymography. Fibroblasts elaborated low levels of MMP-1 under control conditions, and increased amounts following stimulation with keratinocyte culture fluid. Concomitant treatment of fibroblasts with two different EGF receptor antagonists had little effect on keratinocyte induction of MMP-1 synthesis. In contrast, concomitant treatment with recombinant interleukin-1 (IL-1) receptor antagonist suppressed the majority of the stimulating activity in keratinocyte culture fluid. Inhibition was dose-responsive. In additional studies, fibroblasts were treated with HGF or with culture fluid from HGF-treated keratinocytes. HGF by itself did not induce significant MMP-1 production, but culture fluid from HGF-treated keratinocytes was more potent than control keratinocyte culture fluid in stimulating fibroblasts to produce MMP-1. Taken together, these data indicate that the elaboration of MMP-1 in organ-cultured human skin involves interaction between the epidermis and dermis. IL-1 produced in the epidermis may play a role in dermal MMP-1 induction.

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### PIG7 Gene Mutation and the Overexpression of its Gene Product in Extramammary Paget's Disease

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Although extramammary Paget's disease (EMPD) is a grave skin disease and is not so uncommon, little is known about the genetic disorder of its pathogenesis. *c-erb B-2* oncogene has been the only suspect gene involved in its carcinogenesis (probably in tumor promotion) because its product is highly expressed in invasive lesions and in metastatic lesions of EMPD. As for the other important oncogenes or suppressor oncogenes such as *ras*, *p53* or *p16*, however, no gene mutation or expression disorder has been reported. In order to search for other candidate gene(s) involved in EMPD pathogenesis, we compared the mRNA expression level of representative tumor-related genes between EMPD lesional skin and intact skin of the same patient using DNA array technique. We extracted total mRNA from 4 EMPD surgical specimens and their normal counterparts (either the surplus of skin graft or the surgical margin), obtained cDNA by RT-PCR, and performed DNA array analysis (Clontech, CA). Several genes were two-fold or more highly expressed in multiple EMPD lesions compared with normal epidermis. Among them, PIG7 mRNA was overexpressed in 3 among 4 EMPD lesions. PIG7 transcription is induced by *p53* expression and is implied to be involved in the *p53*-induced apoptotic pathway. Since *p53* mRNA expression level is not high in any of those 3 EMPD samples compared with the intact skin specimens, we analyzed PIG7 cDNA mutations among 12 EMPD samples, including the former 4 samples, using PCR-SSCP technique. Briefly, we amplified the translation sequence of PIG7 gene as 53 overlapping fragments (206–216 base pairs), performed SSCP analysis followed by direct sequencing of DNA fragments derived from the shifted bands. As a result, 5 EMPD samples had shifted bands (two of them had point mutations which lead to amino acid substitutions, one had a silent mutation and the other two samples are under investigation). One sample with amino acid substitution overexpressed PIG7 mRNA in the former DNA array analysis. These results indicate that the overexpression of (mutated?) PIG7 expression would be related to EMPD carcinogenesis, and that genetic mutations underlie its overexpression.

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### Calcitonin Gene-Related Peptide (CGRP) has a Dual Regulatory Function in Human Dermal Microvascular Endothelial Cell (HDMEC) Nuclear Factor $\kappa$ B (NF- $\kappa$ B) Activation

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The cutaneous sensory neuron-derived neuropeptide calcitonin gene-related peptide is capable of modulating numerous biological functions of epidermal and dermal cells via the interaction with specific cell surface receptors. Less is known about CGRP effects on HDMEC activities. To address the hypothesis that endothelial cell NF- $\kappa$ B activation in CGRP receptor-expressing HDMEC is directly modulated by CGRP, HDMEC isolated from human foreskins or cells of the endothelial cell line HMEC-1 were stimulated with CGRP (1–1000 nM) alone or in combination with TNF $\alpha$  or IL-1 $\beta$ . The activation of NF- $\kappa$ B was examined by electrophoretic mobility shift assay (EMSA), Western blotting and NF- $\kappa$ B-luciferase reporter gene assays. After 15 min, CGRP dose-dependently stimulated NF- $\kappa$ B activation and nuclear translocation. Specificity and composition of DNA-protein complexes was demonstrated by supershift analysis using antibodies against the NF- $\kappa$ B subunits *p50*, *p65*/RelA, cRel or RelB. The specific CGRP receptor antagonist GCRP<sub>8-37</sub> was capable of antagonizing this CGRP induced NF- $\kappa$ B activation. In addition, CGRP dose-dependently induced luciferase activity in NF- $\kappa$ B-luciferase transfected HDMEC. However, when cells were costimulated with IL-1 $\beta$  or TNF $\alpha$  and CGRP or protein kinase A activators (forskolin), CGRP (100/10 nM) as well as forskolin antagonized the TNF $\alpha$  or IL-1 $\beta$  induced formation of the NF- $\kappa$ B *p50*/*p65* transactivator complex which is presumably mediated by cytosolic stabilization of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$ . Likewise, the TNF-induced activation of HDMEC ICAM-1, VCAM-1 or E-selectin mRNA expression was antagonized by CGRP in similar concentrations. Thus, these novel data suggest that perivascular sensory nerve-derived CGRP by activating high-affinity receptors is capable of differentially regulating NF- $\kappa$ B controlled endothelial cell gene expression which may be of importance for both initiation and termination of an inflammatory response during cutaneous neurogenic inflammation.

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### Collaboration Between MAP Kinase Activation and p16 Deletion in a Tuberous Sclerosis-Related Sarcoma

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Tuberous sclerosis is a common autosomal disorder due to inactivation of the tumor suppressors tuberlin and hamartin. Cutaneous sarcomas arise in 10% of mice heterozygous for tuberlin, and represent the second most common malignancy in these mice after renal cell carcinoma. We established a cell line (Tsc2Ang1) from a cutaneous sarcoma and analyzed signal transduction and tumor suppressor gene profiles. Tsc2Ang1 cells demonstrate tumorigenesis *in vivo* and high level expression of activated MAP kinase *in vitro*. Introduction of a dominant negative MAP kinase leads to abrogation of tumor growth *in vivo* and tumor dormancy. Genomic analysis shows loss of heterozygosity (LOH) of the tumor suppressor *p16*, and no alteration of *p53*. Surprisingly, LOH of tuberlin was not observed. The potent angiogenic factor vascular endothelial growth factor (VEGF) is expressed *in vivo* as determined by *in situ* hybridization. Cutaneous sarcomas may represent the murine homolog of cutaneous angiofibromas. Inhibition of MAP kinase may be a therapeutic target in the treatment of tuberous sclerosis related tumors. Heterozygosity for tuberlin may predispose to deletion of tumor suppressor genes. Finally, this model suggests a collaboration between *p16* inactivation and MAP kinase activation, which may be operative in other human tumors, including cutaneous melanoma.

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### Genetic and Immunohistochemical Studies of Angiofibromas and Collagenomas in Multiple Endocrine Neoplasia Type 1

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Multiple facial angiofibromas and collagenomas commonly occur in adults with multiple endocrine neoplasia type 1 (MEN1), a familial tumor syndrome caused by mutations in the *MEN1* gene. We have previously shown that MEN1 angiofibromas show loss of heterozygosity (LOH) at the *MEN1* locus and that the neoplastic cells in these tumors are located in perivascular clusters. Here, we examined collagenomas for 11q13 LOH and further characterized the cells within angiofibromas and collagenomas using immunohistochemistry. In preliminary LOH studies of skin lesions from 2 MEN1 patients, clusters of potentially neoplastic cells were microdissected from 2 collagenomas and 1 biopsy of normal appearing skin. Four microsatellite markers at the *MEN1* locus (D11S449, D11S480, PYGM, INT-2) showed LOH occasionally in perifollicular stromal cells of collagenomas but not in epidermal cells or normal appearing skin. These studies extend our report that, instead of being hamartomatous, MEN1 angiofibromas and collagenomas contain mono- or oligoclonal neoplastic proliferations of cells. To clarify the anatomic zone of this clonogenic process, 3 angiofibromas and 9 collagenomas from 5 patients with MEN1 were stained for vimentin (mesenchymal cells), factor XIIIa (dermal dendritic cells), CD34 (endothelial cells, stromal cells), CD68 (macrophages, mast cells), S-100 (melanocytes, neuronal cells, Langerhans cells), HMB-45 (melanocytes), and smooth-muscle actin (myofibroblasts, pericytes). Although no clear demarcation of markers was observed with regard to the distribution of LOH, perivascular and perifollicular stromal cells stained positively for vimentin, negatively for smooth-muscle actin, mostly positive for factor XIIIa and occasionally positive for CD34. Compared to normal appearing skin, angiofibromas and collagenomas showed strongly increased dermal staining for vimentin, factor XIIIa, and CD34, and modest increases in CD68 and S-100. In conclusion, genetic and immunohistochemical analyses suggest that MEN1 angiofibromas and collagenomas are neoplastic proliferations of mesenchymal cells showing 11q13 LOH.

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### Basic Fibroblast Growth Factor Stimulates Lef/Tcf-Dependent Transcription in Human Umbilical Vein Endothelial Cells

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Lef/Tcf proteins belong to a family of architectural transcription factors, which control many developmental processes and also play an important role in oncogenesis. Classical activators of Lef/Tcf-dependent transcription belong to the Wnt family of proteins, which translocate  $\beta$ -catenin into the nucleus and allow the formation of transactivation-competent Lef/Tcf/ $\beta$ -catenin complexes. Here we show that in human endothelial cells, also FGF induces nuclear translocation of  $\beta$ -catenin, which enhances transcription of a Lef/Tcf reporter construct by two fold. Performing semiquantitative RT-PCR analysis of Wnt mRNAs we show that this FGF effect is not mediated by induced Wnt expression. Since down-regulation of the glycogen synthase kinase-3 (GSK-3) activity is the prerequisite of  $\beta$ -catenin translocation into the nucleus we performed kinase assays. Like Wnt proteins, FGF induced a rapid reduction of GSK-3 activity. This was completely blocked by the MEK kinase inhibitor UO126 (1  $\mu$ M) back to baseline, indicating that FGF acts on GSK-3 via the MAP kinase pathway. Employing gel shift assays we show that FGF enhances the binding of a Lef/Tcf/ $\beta$ -catenin complex to a Lef/Tcf binding site within the promoter of the urokinase receptor. In summary, we describe a novel modulatory mechanism of  $\beta$ -catenin-mediated transcription in endothelial cells controlled by FGF, which is independent from Wnt proteins.

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### Overexpression of Bcl-2 in an *In Vivo* Model of Low Grade Angiosarcoma

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Bcl-2 expression in normal vascular endothelial cells (EC) promotes vascular maturation without overt tumor formation. An *in vivo* model of low-grade angiosarcoma was developed, and utilized to evaluate the effects Bcl-2 in combination with a transforming gene. The SV40 transformed murine endothelial cell line MS-1 was suspended in a collagen/fibronectin matrix. This resulted in the *in vitro* formation of vascular cords. When implanted subcutaneously into SCID-beige mice, tumors composed of dense networks of perfused vascular structures, containing EC with hyperchromatic nuclei, and intravascular endothelial hyperplasia, were observed. Retroviral transduction was utilized to overexpress a caspase resistant form of Bcl-2, or the control transgene EGFP, in the EC incorporated into these constructs prior to implantation in mice ( $n=9$ ). Thirty days after implantation 5 mice in each group were harvested. The morphology of the resultant vascular networks in both groups was similar to those which had not been transduced. Although the mean Bcl-2 transduced tumor volume ( $71.9 \pm 19.7 \text{ mm}^3$ ) was greater than those transduced with EGFP ( $34.0 \pm 26.5 \text{ mm}^3$ ) at 30 days, 60 days after implantation, there was no significant volume difference between the Bcl-2-transduced ( $60.2 \pm 23.3 \text{ mm}^3$ ) and the EGFP-transduced ( $77.5 \pm 19.65 \text{ mm}^3$ ) tumors ( $p=0.78$ ,  $n=4$ ). No gross or microscopic metastases were detected in either group. Local invasion was minimal, although slightly greater extension of the atypical vascular structures into the abdominal wall musculature was observed in the Bcl-2 transduced tumors. In summary, suspension of MS-1 cells in a tissue like matrix resulted in the *in vivo* formation of tumors resembling low grade angiosarcomas, but concomitant overexpression of Bcl-2 did not significantly increase the tumorigenicity of these cells. These findings suggest that overexpression of Bcl-2 in normal EC may be a safe modification when applied to tissue engineering.

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### Heterogeneity of the $\gamma$ -T-Cell Receptor ( $\gamma$ TCR) is Accompanied by a Heterogeneous Profile of the Lymphoid Infiltrate in Vulvar Lichen Sclerosus (LS) and Associated Squamous Cell Carcinomas (SCC)

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The lymphoid infiltrate in LS has been shown to be of a T-cell phenotype with a high percentage of  $\gamma$ TCR rearrangement. We evaluated the differences of the immunohistochemical (IHC) phenotype between  $\gamma$ TCR-positive and  $\gamma$ TCR-negative LS cases and tested if  $\gamma$ TCR-rearrangement would be a prerequisite for the development of a SCC in LS. We analyzed 15 SCC arising in LS, 15 SCC without LS, and 15 LS by PCR for  $\gamma$ TCR and compared the IHC profile of the lymphoid infiltrate (CD2, CD3, CD4, CD8, CD20, CD57, fascin, and granule-associated cytotoxic proteins granzyme, TIA and perforin). Clonally rearranged  $\gamma$ TCR was identified in 5/15 LS, 6/15 SCC with LS and in 0/15 SCC without LS. Analysis of multiple concomitant biopsies of a single lesion of LS and various regions of the SCC, however, demonstrated a heterogeneity of the  $\gamma$ TCR within a single lesion. All LS covered by intact mucosa/epidermis showed a predominant CD8-positive dermal and epidermal lymphoid infiltrate with TIA positive dermal lymphocytes. In ulcerated LS, CD4-positive T-cells predominated. Differences between  $\gamma$ TCR-positive and  $\gamma$ TCR-negative biopsies were observed within submucosal and perivascular lymphocytic cell aggregates (LA), which were predominantly positive for CD20 and TIA in  $\gamma$ TCR-positive with numerous fascin-positive cells in the B-cell fraction, and individual CD8, CD57, granzyme and perforin-positive cells in the peripheral zones of the LA. LA in  $\gamma$ TCR-negative biopsies demonstrated only single peripherally located CD20 and fascin-positive lymphocytes, but a diffuse CD57 and granzyme staining in the absence of perforin staining. We postulate that development of T-cell clones in lesional skin of vulvar LS is a focal event, which is not required for the development of a SCC. The presence of B-cells, fascin and perforin-positive cells in  $\gamma$ TCR-positive biopsies of LS has not been previously described and may represent an immunologic mechanism to exert a cellular cytotoxicity to the proliferating T-cell clones.

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### Loss of Heterozygosity (LOH) is a Quantitative Trait in Acquired Melanocytic Nevi and can Help to Discriminate Between Benign and Dysplastic Lesions

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Loss of heterozygosity (LOH) is frequently found in acquired melanocytic nevi and malignant melanomas. Its role in melanoma development thus remains unclear although LOH is a sign of genetic instability and should therefore facilitate malignant progression. To assess the role of LOH as well as microsatellite instability (MSI) in nevi and melanomas we used a quantitative approach by analysing multiple microdissected tumor areas arrayed linearly within a histological tissue section. Six benign nevi, 6 dysplastic nevi and 5 malignant melanomas were analyzed at microsatellite markers D9S162 and D14S53. Each microdissected region contained about 500 cells and the mean number of microdissected areas per tumor was 9. LOH and MSI were demonstrated by electropherogram analysis using an automated sequencer (ABI Prism 373). For LOH and MSI, the frequency of mutants (FM) as well as the frequency of distinguishable tumor cell clones (FC) were then used to calculate a mutation index (MI):  $MI = (FM \times FC)^{0.5}$ . For LOH, MI was significantly higher in dysplastic nevi than in benign acquired nevi at both markers (D9S162:  $0.48$  vs.  $0.17$ ,  $p=0.004$ ; D14S53:  $0.43$  vs.  $0.19$ ,  $p=0.05$ ). A similar but statistically not significant trend was observed for MSI. Melanomas did not differ from dysplastic nevi with regards to MI. Our data suggest that quantitative LOH analysis may be useful to discriminate on a molecular basis between benign and dysplastic acquired nevi.

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### The Kaposi's Sarcoma Associated Herpesvirus G Protein-Coupled Receptor Up-Regulates VEGF and Promotes Endothelial Cell Survival

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The Kaposi's Sarcoma Associated Herpes Virus (KSHV) is the infectious etiologic agent of Kaposi's Sarcoma (KS), a multifocal neovascular neoplasm frequently associated with AIDS. One of the viral genes, the KSHV G protein-coupled receptor (KSHV-GPCR), is a key molecule in the pathogenesis of KS, playing a central role in the promotion of VEGF-driven angiogenesis and spindle cell proliferation. We have studied the signal transduction pathways controlling VEGF expression and endothelial cell survival induced by the KSHV-GPCR. We found that this viral receptor enhances the expression of VEGF by stimulating the activity of HIF-1 $\alpha$ , which activates transcription from a hypoxia response element within the VEGF promoter. Stimulation of HIF-1 $\alpha$  by the KSHV-GPCR involves the phosphorylation of the regulatory/inhibitory domain of the protein by the p38 and MAPK signaling pathways, enhancing HIF-1 $\alpha$  transcriptional activity. Moreover, specific inhibitors of the p38 (SKF86002) or MAPK (PD98059) pathways were able to inhibit the transactivating activity of HIF-1 $\alpha$ , as well as VEGF expression and secretion in cells overexpressing this receptor. We further found that the KSHV-GPCR is able to promote endothelial cell survival and that this ability is not dependent on the receptor's induction of VEGF secretion. When expressed in human primary endothelial cells, the KSHV-GPCR is able to rescue cells from apoptosis induced by serum deprivation. Interestingly, expression of the KSHV-GPCR also potently induced the kinase activity of Akt/PKB, a critical molecule involved in the control of cell survival and in tumor development. Indeed, we found that the PI3K-Akt pathway is required for the prevention of cell death induced by the KSHV-GPCR, as both treatment with wortmannin and expression of a dominant negative interfering mutant of Akt are sufficient to abolish the inhibition of apoptosis in response to the expression of the KSHV-GPCR. These results suggest that the KSHV-GPCR could promote the survival and uncontrolled cell proliferation of the viral-infected cells and may help to explain the molecular mechanism(s) whereby viral and cellular oncogenes govern the acquisition of the angiogenic phenotype and drive tumor-induced neovascularization.

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### Cumulative and Conditional Probabilities for Death from Squamous Cell Carcinoma (SCC) in Recessive Dystrophic EB (RDEB): Experience of the National EB Registry (NEBR), 1986-2000

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SCCs are a common occurrence in RDEB, especially in those patients with the Hallopeau-Siemens subtype. It is also known that metastases and death do occur in some of these patients, despite early diagnosis and aggressive surgical treatment of these tumors. We have attempted to quantitate the cumulative and conditional risks for death from SCC in RDEB, using life table analysis of 14 years' data, collected through 12/31/00, on all RDEB patients who have been enrolled in the NEBR. We have shown that the cumulative risk of developing a first SCC in RDEB-HS is nearly 6% by age 20, rising rapidly to nearly 81% by age 40. Using the same study population, the cumulative risk of death from any SCC was found to be 4.2%, 8.6%, 21.4%, 29.9%, 57.9%, and 70.0% by age 20, 25, 30, 35, 40, and 45, respectively, with the highest conditional risk (40.0%) occurring between ages 35-40. In contrast, a much lower cumulative risk was found in patients with the non-Hallopeau-Siemens subtype (RDEB-nHS), beginning at 2.2% by age 25, and progressing to 5.4%, 8.4%, and 15.4% by ages 25, 45, and 55, respectively. These latter findings are consistent with the overall lower cumulative risk of SCC in RDEB-nHS (only 17.4% and 43.1% by ages 40 and 55), compared to RDEB-HS. Based on these data, it can be concluded that the risk of fatality from SCC is very high in patients with RDEB-HS, and that this usually occurs within about 5 years of the diagnosis of the first SCC in these patients. A much lower but still significant risk of death from SCC occurs in patients with RDEB-nHS, with a much longer period of time from first diagnosis to subsequent death. Based on these findings, we recommend biopsy of any suspicious lesions in RDEB that arise on or after about age 10.

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### Frequency and Cumulative Risk of Cutaneous Squamous Cell Carcinomas in Inherited Epidermolysis Bullosa within the United States: Experience of the National EB Registry (NEBR), 1986-2000

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Although it has been known that squamous cell carcinomas (SCCs) may arise in some types of EB, the magnitude of this risk was first demonstrated in 1995 by analysis of data collected by the NEBR, a nationally based, cross-sectional and longitudinal epidemiologic project, based on 9 years' data on its first 1900 patients. At that time, the cumulative risk of developing at least 1 SCC in Hallopeau-Siemens RDEB (RDEB-HS) by age 40 was estimated by life table technique to be 53%. We now report on our experience, through 12/1/00, on 2629 consecutively enrolled patients. Within this cohort, 0.36%, 0.90%, 1.08%, and 13.11% of all NEBR enrollees with EB simplex (EBS), junctional EB (JEB), dominant dystrophic EB (DDEB), and RDEB had developed at least 1 SCC. Significant differences were further noted when these data were stratified by major EB subtype. Life table analyses confirmed that SCCs were primarily the concern, during at least mid-adulthood, of patients with RDEB, although rare cases were also found in JEB. By age 40, the cumulative risk of at least 1 SCC was 0.39%, 18.18%, 0.00%, 0.00%, 80.80%, and 17.41% in EBS, JEB-H, JEB-nH, DDEB, RDEB-HS, and RDEB-nHS, respectively. By age 50, these cumulative risks had risen to 0.64%, 18.18%, 4.76%, 1.83%, 86.29%, and 38.17%, respectively. When the ratios of these cumulative risks were compared, patients with RDEB-HS, RDEB-nHS, and JEB were found to be 207.2, 44.6, and 5.1 times, respectively, more likely to have developed at least one SCC than patients with EBS by age 40. These strikingly higher risks for development of SCC suggest the near universal risk of SCC by age 50 in RDEB-HS, as well as clinically substantial risks for patients with RDEB-nHS and both subtypes of JEB.

## 619

**Basal Cell Carcinomas and Malignant Melanomas in Inherited Epidermolysis Bullosa (EB) within the United States: Experience of the National EB Registry (NEBR), 1986–2000**

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We report on the occurrence of basal cell carcinomas (BCCs) and malignant melanomas (MMs), through 12/1/00, on 2629 patients who have been followed by the NEBR, a nationally based, cross-sectional and longitudinal project. Differences between current data and those by the NEBR in 1995 reflect increased numbers of subjects, longer follow-up, and, in the case of RDEB, more precise clinical and molecular subtyping. Within the latest cohort, only 1.76%, 0.00%, 1.08%, and 0.52% of all enrollees with simplex (EBS), junctional (JEB), dominant dystrophic (DDEB), and recessive dystrophic (RDEB) EB had developed one or more BCC. Similar frequencies of BCCs (1.51–1.90%) were seen within the two major EBS subtypes. Within the RDEB subjects, BCCs occurred exclusively within those with non-Hallopeau-Siemens (RDEB-nHS) disease. Lifetable analysis demonstrated that the onset of BCC in EBS-nWC began between ages 30–35, with cumulative risks of 0.58%, 2.08%, 4.44%, 8.09%, and 17.28% by ages 35, 40, 50, 60, and 70, respectively. A similar pattern was noted in EBS-WC, although the earliest onset was by age 45–50, with a cumulative risk of 2.19% by age 50, reaching 14.93% by age 70. Only 2 of 252 RDEB-nHS patients developed BCCs (and only in late adulthood), and by age 60 the risk of BCC in DDEB was only 0.67%. The cumulative risk of MM by age 50 was low in EBS (0.57%) and DDEB (1.36%) and absent in JEB. The risk of MM in RDEB, at least through age 65, was confined to RDEB-HS, with cumulative risks of 0.82%, 1.71%, and 2.76% by ages 4, 7, and 12. These data demonstrate that BCC is not a clinically significant concern in any type of EB until at least mid-adulthood, whereas MM, although a rare event, is a real risk in children with RDEB-HS. The latter strongly suggests the need for careful surveillance for pigmented lesions in patients with RDEB, beginning in early childhood.

## 621

**DNA Homologous to the 3' Telomere Overhang Mimics UV Induced Cytokine Responses in Keratinocytic Cells**

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Single-stranded DNA fragments partially (or totally) homologous to the telomere sequence TTAGGG induce cellular DNA damage responses, such as melanogenesis and apoptosis, suggesting that exposure of the 3' telomere overhang during DNA damage and/or repair is a physiologic stimulus for such responses. UV irradiation is a DNA-damaging agent that induces cytokine synthesis and secretion in keratinocytes (Kc), and we have shown that tumor necrosis factor and IL-10 are induced by thymidine dinucleotide. To determine if IL-1 is similarly regulated, well-differentiated squamous carcinoma cells (SCC12F) were stimulated with an 11-base oligonucleotide (11mer-1) homologous to the telomere overhang. Controls were treated with diluent alone or with the complementary sequence (11mer-2). Duplicate cultures were exposed to 30 mJ per cm<sup>2</sup> solar simulated light or were sham irradiated (CTL). Within 2 h there was 1300% increase of IL-1 mRNA in UV compared to CTL cells. The induction was sustained through 48 h when the experiment was terminated. Western blot revealed that IL-1 protein level was ~300% that of CTL cells at least through 96 h. Moreover, within 6 h, IL-1 protein level in medium conditioned by UV irradiated cells was 38 ± 2 pg per ml vs. = 1 pg per ml in sham irradiated conditioned medium ( $p < 0.03$ , non-paired *t* test). These data demonstrate that a DNA oligomer homologous to the 3' telomere overhang induces IL-1 similar to physiologic UV irradiation. The data suggest that exposure of telomeric DNA during UV-induced damage and/or repair is a potent stimulus for the immunomodulatory response following UV irradiation of skin. Homologous oligomers may prove therapeutic for UV-responsive dermatoses.

## 623

**Genomic Analysis Reveals a Coordinate Transcriptional Program Underlying Responses of Human Keratinocytes to UVB Radiation**

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UVB radiation is an important inducer of many biological changes in skin, of which keratinocytes are a key target. To gain better insight into changes in gene expression generated in the early phase after UVB exposure, we took advantage of complementary RNA (cRNA) microarray hybridization to analyze mRNA expression in cultured keratinocytes isolated from freshly excised neonatal human foreskin by comparing differences in UVB-irradiated (single dose of 100 J per m<sup>2</sup>) vs. sham-irradiated cells. Six hours after UVB- or sham-irradiation, total RNA was isolated from keratinocytes, and then cRNA was synthesized and hybridized to a GeneChip expression array (Affymetrix) consisting of 6800 genes. Based on a threshold of 2-fold change, 187 genes (2.8%) were designated to be UVB-responsive. Surprisingly, none of these genes were shown previously to be modulated by UVB. And conversely, several genes represented in the microarray that had been reported previously to be UVB-responsive showed little (< 2-fold) to no change. Clustering based on known functions indicated that among 88 up-regulated genes, 9 encode for cytochrome c subunits, 6 for ribosomal proteins, and 2 for regulators of apoptosis. By contrast, many of the 99 down-regulated genes are involved in cell differentiation and transport. Finally, Northern blotting was used to validate results. For UVB-responsive genes examined to date, there has been concordance with respect to the direction of UVB regulation (up or down). We conclude that human keratinocytes respond to UVB radiation (6 h after a single low dose) through a coordinate transcriptional program, enhancing several translation systems while also suppressing differentiation and transport. These findings affirm the utility of genome-wide analyses for studying effects of UV radiation on skin cells.

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**A Mouse Model for UV-Induced Junctional Melanoma**

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Investigations of malignant melanoma have been hampered by lack of an animal model with histology and pathogenesis similar to human disease. We used melanoma-prone transgenic mice ectopically expressing hepatocyte growth factor/scatter factor (HGF/SF) as an experimental model system to ascertain the effects of UV radiation on melanomagenesis. HGF/SF stimulates growth, motility and morphogenetic transformation in cells, including melanocytes, expressing its tyrosine kinase receptor, c-Met. HGF/SF transgenic mice have ectopic extra-follicular localization and accumulation of melanocytes in the dermis, epidermis and junction and, if untreated, developed cutaneous melanomas of dermal pathology with a mean onset age of 21 months. Transgenic mice and wild type littermates given UV radiation (FS40 sunlamps, UV doses 2.25–6.0 kJ per m<sup>2</sup>) 3 times weekly developed cutaneous tumors with a mean onset of 26 and 37 weeks, respectively ( $p < 0.001$ ). All tumors were sarcomas or carcinomas. The conspicuous absence of melanocytic tumors occurred despite immunohistochemical detection of UV-induced stimulation ( $p < 0.001$ ) of melanocyte proliferation and a 2.6- and 4.6-fold increase in melanocyte numbers in the dermis and epidermis, respectively. In contrast, neonatal transgenic and wild type littermates given a single dose of UV radiation (FS40, 9 kJ per m<sup>2</sup>), had a high incidence of premalignant nevus-like lesions, progressing to malignant melanoma and metastasis with an estimated cumulative tumor rate of 70% and a median tumor time of 9 months. An additional UV dose (18 kJ per m<sup>2</sup>) at 6 week of age did not accelerate melanoma formation but increased the number of melanocytic lesions/animal ( $p < 0.03$ ). Remarkably, 75% of UV-induced melanocytic lesions developed in apposition to epidermal elements and had a junctional morphology. Melanomas were reminiscent of human radial and vertical growth phase and Pagetoid melanomas. Molecular studies showed expression of HGF/SF in melanocytes followed by induction of c-met RNA in nevus lesions, suggesting an autocrine HGF/SF-c-Met loop as an early step in melanoma formation. Mutational studies are underway. This novel model of UV-induced melanoma provides a much-needed experimental tool to facilitate analysis of the photobiology and genetics of melanoma and the development of effective prevention and treatment strategies.

## 622

**Induction of Melanogenesis *In Vivo* by UV Light and Oligonucleotides is Mediated by p53**

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DNA oligonucleotides that share sequence homology with the telomere 3' overhang sequence, TTAGGG, induce DNA damage responses when added to mammalian cells. Specifically, we have shown that thymidine dinucleotide (pTT), a partial telomere homology, as well as ultraviolet light enhances tyrosinase expression and melanin content in p53 proficient cells while after pTT or UV melanoma cells with dominant negative p53 show no up-regulation of tyrosinase, the rate-limiting enzyme for pigmentation. To determine the role of p53 in these tanning responses, we designed two 11 base oligonucleotides, one homologous to the telomere overhang sequence (11mer-1) and an unrelated control (11mer-2). In p53 proficient and p53 knock-out C57/B16 mice we treated ears topically with 100 μM pTT or 40 μM 11mer-1 or –2 or diluted alone five days a week or UV-irradiated three times a week, for three weeks. Three days after the last treatment, the ears were removed, sectioned and stained with Fontana Masson to assess melanin content by image analysis. Compared to controls, there were increases in UV-irradiated ears of both p53+/+ and p53–/– mice ( $p = 0.001$ ) but the increases were greater in p53+/+ than in p53–/– mice ( $p = 0.003$ ): (625% vs. 260%). Compared to diluent controls, in p53+/+ mice, both oligomers increased melanin content ( $p = 0.001$ ), but 120% for pTT and 630% for 11mer-1; but there was no increase in p53–/– mice treated with either oligomer. The control 11mer-2 had no significant effect in either animal group. The data demonstrate that pigmentation induction by oligonucleotides that share sequence homology with the telomere 3' end is totally dependent on p53 and that pigmentation induced by UV is substantially p53 dependent. The data suggest that tanning is largely a direct DNA damage response and can be mediated by exposure of telomere 3' overhang DNA.

## 624

**DNA Oligonucleotides Modulate DNA Repair, Cell Cycle and Apoptosis: Therapeutic Implications**

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Ultraviolet (UV) irradiation produces DNA photoproducts and if not repaired, can cause mutations. UV-induced DNA damage delays cells for entering into the S phase, hence, increases repair time; in case of severe damage they undergo apoptosis. We have shown that DNA oligomers mimic many effects of UV irradiation, based on their homology to the telomeric sequence TTAGGG. We therefore examined the effect of thymidine dinucleotide, pTpT, and 5' phosphorylated oligonucleotide (p9mer) on the cell cycle, and baseline as well as UV-induced expression of DNA repair and apoptosis proteins in human fibroblasts (fb). To determine if pretreatment with oligomers alters these cellular responses, we pretreated fb with 100 μM pTpT or 40 μM p9mer or diluent (DME) for 48 h, then UV irradiated (30 mJ per cm<sup>2</sup>). Treatment with oligomers up-regulated baseline levels of DNA repair proteins PCNA 400–600%, ERCC1200–1000%, RPA 300–1000%; XPA 1500–2200%; p53 400–600%, p21 150–600, MDM2230–350%; Bax 130–150% proteins. Compared to diluent oligomers treated cells revealed significant reduction 150–400% in cell counts at 24 and up 72 h 300–400%. FACS analysis showed fb treated with oligomers remained in Go/G1 phase up to 24 h (p9mer) or 48 h (pTpT), whereas diluents treated cells grew exponentially. A greater number of p9mer treated cells showed annexin V and activated caspase-3 positivity (35–42%) compared to pTpT (16–23%) or diluent (11–19%) treated cells at 48 and 72 h. Furthermore, the rate of removal of thymine dimers showed that cells pretreated with oligomers and then UV irradiated removed photoproducts 40–60% more efficiently at 16 and 24 h, respectively. These data confirm the ability of oligomers to evoke DNA damage responses in human cells, with potency dependent on degree of telomere homology. Such oligomers might be employed to endogenous DNA repair capacity and to reduce the carcinogenic risk from UV irradiation, as well as for “sunless phototherapy” of various dermatoses.

## 625

**UVB Stimulates the COL7A1 Promoter via the NIK-IKK-NF- $\kappa$ B Pathway Independent of the TNF- $\alpha$  Receptor**

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Type VII collagen is the major if not the only component of the anchoring fibrils which connect the basal membrane zone with the underlying dermis. UVB has been attributed not only with an enhanced skin cancer rate, but also to be a strong inducer of solar elastosis and causing accumulation of extracellular matrix proteins in the skin. Recently, Chen and colleagues described an strong enhancement of Type VII Collagen mRNA after UV irradiation. We therefore wanted to elucidate the mechanism of UV induced COL7A1 activity by the usage of a series of 5' deletions of the human Type VII collagen gene promoter in transient cell transfections. An up to 9-fold increase after UVB irradiation of 772 basepairs of the promoter linked to a cat reporter gene could be observed. To further finemap the region in the promoter responsible for mediating this effect, we used created 5' deletions and could identify a region between -252 and -230 as response element. This 22 basepair element contains a functional NF- $\kappa$ B binding site. Introduced mutations in this site caused a strongly diminished response to UVB irradiation. The role of NF- $\kappa$ B was further supported by electrophoretic mobility shift assays in which binding of nuclear proteins from UVB-irradiated fibroblast cultures to the response region of the promoter is competed by consensus NF- $\kappa$ B oligonucleotide, but not by AP-2 or Sp-1 oligonucleotides. Key components of the intracellular signal transducing pathway which regulates NF- $\kappa$ B activation are NIK and the IK-B kinases. Therefore their role in the NF- $\kappa$ B-mediated stimulation of COL7A1 was investigated. Cotransfection of a dominant negative NIK expression vector with -252 COL7A1 prevented activation of the type VII collagen gene promoter by UVB. Similar results were obtained when using a dominant negative IKK expression vector, indicating that intact NIK and IKK cascade are required for the intracellular signaling and resulting UVB induced NF- $\kappa$ B mediated activation of COL7A1. In contrast, overexpression of a dominant negative TNF- $\alpha$  receptor did not interfere with UVB-driven activation of the COL7A1 promoter. In conclusion, this is the first study which demonstrates an involvement of the NIK-IKK-NF- $\kappa$ B pathway in UVB mediated activation of the human type VII collagen gene promoter independent of the TNF- $\alpha$  receptor in dermal fibroblasts.

## 627

**IL-12 Protects from UV-Induced Apoptosis by Enhancing DNA Repair**

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Induction of apoptosis of keratinocytes by ultraviolet radiation (UV) is a protective phenomenon relevant in limiting survival of cells with irreparable DNA damage. Hence, alterations of UV-induced apoptosis may have significant impacts on photocarcinogenesis. Based on the previous observation that the pro-inflammatory cytokine interleukin (IL)-1 enhances UV-induced apoptosis, we screened several other mediators for their effects on UV-induced apoptosis. Pretreatment of the keratinocyte cell lines HaCaT and KB with the immunomodulatory cytokine IL-12 led to a significant reduction in cell death following UV exposure, as shown by the use of a DNA fragmentation ELISA and annexin-V staining. Surprisingly, IL-12 treatment caused a remarkable reduction in UV-specific DNA lesions, as determined by South-western dot blot analysis using antibodies directed against UV-specific pyrimidine dimers. Spectrophotometric analysis excluded a filtering effect of IL-12. Comet assays revealed longer comets in UV-exposed cells which were treated with IL-12. This finding suggests that IL-12 may enhance DNA repair. RNase protection assays revealed that the expression of 3 components of the nucleotide excision repair complex (RPA, DBB1, DBB2) was induced by IL-12. To determine the *in vivo* significance of these findings, IL-12 was injected i.c. into skin areas of BALB/c mice which were subsequently exposed to UV. Immunohistochemical analysis revealed remarkably fewer UV-specific DNA lesions in the epidermis of mice which had been subjected to IL-12 treatment. Accordingly, the number of sunburn cells was significantly reduced upon IL-12 treatment. Taken together, this is the first demonstration of a cytokine being able to protect cells from apoptosis induced by a DNA damaging agent via modulation of DNA repair.

## 629

**Array of Sun: UVB-Regulated Genes in Epidermal Keratinocytes**

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Epidermis, while nobly protecting the rest of our body, bears the majority of photodamage. Hypothesizing that the skin has developed elaborate mechanisms to protect itself from the UV light, we used DNA array technology to follow changes in gene expression in response to UV in epidermal keratinocytes. Of the 6800 genes represented on Affymetrix chips, UV light regulates approximately 320 by 2.5 fold or more within the first 24 h after illumination. Three waves of regulated genes, at 0.5–2, 4–8, and 16–24 h post treatment can be distinguished. Genes regulated in the first wave include transcription factors and signal-transducing proteins that change cell phenotype. The early induction of mitochondrial proteins and glycolytic enzymes provide cells with a burst of energy, while several energy requiring proteins, such as transporters, are suppressed. The second wave notably includes secreted growth factors, cytokines and chemokines; keratinocytes, having accomplished the immediate changes in their own physiology, alert the cells in the surrounding tissues to the damage. The third wave prominently includes the components of the cornified envelope; keratinocytes attempt to enhance the protective covering of the epidermis and, not coincidentally, terminally differentiate and so die. During the 24-h period, UV light induces the expression of histones and enzymes that synthesize dNTPs, raw materials for DNA synthesis. Detoxifying and antioxidant enzymes, RNA processing proteins and translation facilitators are also induced by UV light. Using a novel skin organ culture model we demonstrated that the UV-induced changes in gene expression detected in keratinocyte cultures also occur in human epidermis *in vivo*. In summary, human epidermal keratinocytes in response to UV commence DNA repair, alter their assortment of transcription factors and other signal transducing proteins, procure more energy, alert the surrounding tissue and differentiate.

## 626

**UVB-Irradiated Dendritic Cells (DC) Induce Nonproliferating, Regulatory Type T Cells (TC)**

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It is well established that low-dose UVB-radiation (UVBR) inhibits the APC function of murine Langerhans cells *in vivo* and converts them from immunogenic to tolerogenic APC. Recently, we have shown that UVB-irradiated murine bone marrow-derived DC (UVB-DC) suppressed proliferation of naive and primed TC, but tolerized primed TC only. To examine the underlying mechanism for these differences, naive OVA<sub>323-339</sub>-peptide-specific, TCR-transgenic TC from DO11.10 mice were analyzed following coculture with unirradiated DC or UVB-DC. First, we found UVB-DC to inhibit OVA-specific TC-proliferation UVB dose- and antigen dose-dependently. Analysis of TC cocultured with both, unirradiated DC and UVB-DC, revealed an activated TC-phenotype with increased expression of CD25 and CD69 by FACS. Supernatants harvested from cocultures with UVB-DC showed reduced levels of IFN- $\gamma$ , IL-2, and IL-4, but not TGF- $\beta$ , compared to unirradiated DC as determined by ELISA. Furthermore, these TC did not proliferate upon restimulation. Interestingly, addition of these nonproliferating TC to cocultures of naive TC and freshly prepared unirradiated DC inhibited TC-proliferation depending on the number of added nonproliferating TC. Also, in supernatants increased levels of TGF- $\beta$  were found. Therefore, our data indicate that UVB-DC propagate TC with a regulatory function. Since regulatory TC are characterized by enhanced TGF- $\beta$ -secretion and increased CTLA-4-expression, we currently investigate the role of CTLA-4 phenotypically and functionally. In conclusion, we have shown UVB-DC to inhibit proliferation of naive OVA-specific TC. These TC, exhibiting an activated phenotype and increased TGF- $\beta$ -production, suppress proliferation of naive TC cocultured with unirradiated DC. These results suggest that UVB-DC induce nonproliferating, regulatory type TC.

## 628

**Spatially Specific and Gene-Specific Targeting of Photodynamic DNA Damage**

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Most methods for studying and treating intact skin do not readily discriminate among the structurally and functionally distinct distribution of cutaneous tissue layers and cells. We have previously used triple helix-forming oligonucleotides linked to psoralen (psOTFOs) to damage specific nucleotide sequences of the human collagenase gene. This method has now been combined with laser-induced two-photon excitation (TPE) to damage a specific nucleotide sequence of DNA residing in a specific location in three-dimensional space. First, to demonstrate TPE-induced photoadducts, collagenase DNA target sequences were incubated with psOTFOs and then irradiated in liquid solution with pulsed 765 nm laser light which is half of the quantum energy needed for conventional one photon excitation used in psoralen + UVA (PUVA) therapy. The DNA target acquired strand-specific psoralen monoadducts in a light dose-dependent fashion. Second, to simulate DNA damage in a tissue, the DNA-*psOTFO* mixture was formed as a cubic centimeter polyacrylamide gel that was irradiated with a converging laser beam targeting the rear of the gel. The largest number of photoadducts formed at the rear of the gel while sparing DNA at the front of the gel, demonstrating spatial localization of sequence-specific DNA damage by TPE. Third, to assess whether TPE treatment could be extended to cells, cultured monolayers of normal human dermal fibroblasts were incubated with tritium-labeled psoralen, without TFO in order to maximize detectable damage, and irradiated by laser TPE. DNA from cells treated with psoralen and irradiated had a four- to seven-fold increase in tritium-activity relative to untreated controls. Functional survival assays indicated the psoralen-TPE treatment was no more toxic than psoralen alone. These results are proof of principle that DNA damage can be simultaneously manipulated both at the nucleotide level and in three-dimensional space. This approach should be useful for dissecting whole tissue responses to DNA damage as well as for gene-specific photodynamic therapy of skin.

## 630

**OGG1-Deficient Cells are Hypersensitive to UVA, but Not to UVB**

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UVA might play an important role in photocarcinogenesis and oxidative DNA base damage has been suggested to be responsible for its mutagenic and carcinogenic properties. Oxidative DNA base lesions are processed by base excision repair (BER), in which a DNA-glycosylase with specificity for a particular kind of base modification removes the damaged DNA base and leaves an abasic AP-site for further processing by AP-endonuclease 1, polymerase- $\beta$ , and DNA ligase. OGG1 is a DNA-glycosylase/AP-lyase, which excises the mutagenic oxidative lesion 8-oxoguanine (8-oxoG) from DNA. Recently, we reported that human fibroblasts, in which BER is blocked by methoxyamine are hypersensitive to UVA and that CHO cells overexpressing APE-1 are hypersensitive to UVA. We can now report that this altered UV-sensitivity is limited to the UVA-range, because sensitivity to UVB was found not to be altered. In order to further clarify the role of oxidative DNA damage and its repair in the biological response to UVA, we studied UVA1- and UVB-induced cytotoxicity in embryonal fibroblasts from OGG1<sup>-/-</sup> and OGG1<sup>+/+</sup> mice. OGG1<sup>-/-</sup> cells were shown to have a deficient global genome repair of 8-oxoG, but an intact transcription coupled repair. Irradiation of wildtype cells with 5 or 10 J per cm<sup>2</sup> UVA1 (340–420 nm) did not slow their proliferation. In contrast, the OGG1<sup>-/-</sup> cells exhibited a significant inhibition of cell proliferation by these UVA1-irradiations, with reduced cell yields (as compared to the unirradiated controls) ranging from 57 to 29% with 5 J per cm<sup>2</sup> UVA1 and 38–16% with 10 J per cm<sup>2</sup> UVA1 at days 1, 2, and 3 after irradiation. The sensitivity of these two cell lines to UVB (10 or 25 mJ per cm<sup>2</sup>) was not different. This isolated hypersensitivity of OGG1<sup>-/-</sup> cells to UVA1, but not to UVB, demonstrates that unrepaired UVA-induced oxidative DNA damage in nontranscribed genes decreases cell survival and/or proliferative capacity. Together with our prior data, this underlines a pivotal role of oxidative DNA damage and BER in the cellular response to UVA.

## 631

### Oxidative Inhibition of Membrane-Associated Protein Tyrosine Phosphatase Activates EGF-Receptors, which are Critical Mediators of the UV Responses in Human Keratinocytes

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Ultraviolet (UV) irradiation rapidly activates epidermal growth factor receptors (EGFR) in human keratinocytes (KC). We have investigated the role of EGFR in cellular responsiveness to UV and the mechanism of UV activation of EGFR. Treatment of human skin *in vivo* with genistein, an EGFR inhibitor, reduced UV-induced EGFR activation by 75%, and blocked subsequent activation of ERK and JNK MAP kinases by 50%. Genistein also inhibited UV-induction of c-Jun protein and collagenase mRNA by 50% and 60%, respectively. Treatment of intact KC with the specific EGFR inhibitor PD169540 reduced UV activation of ERK by 80%, JNK by 30%, and completely blocked induction of both c-Fos and c-Jun mRNA, indicating that all of these cellular responses are dependent on EGFR. EGFR in isolated KC membranes, unlike intact cells, was not activated by UV irradiation (10–40 mJ per cm<sup>2</sup> UVB source), indicating that UV does not act directly on EGFR. In contrast, addition of peroxide or protein tyrosine phosphatase (PTP) inhibitors activated EGFR 10-fold in isolated KC membranes. Coincident with activation of EGFR, peroxide caused significant inhibition of membrane-associated PTP activity. PTP activity coimmunoprecipitated with EGFR, and this activity was substantially reduced following UV irradiation of intact KC. In summary, EGFR mediates many critical UV-induced cellular responses, including MAP kinase activation, induction of transcription factor AP-1, and gene expression. Our data indicate that EGFR is constitutively phosphorylated, and maintained in an inactive, dephosphorylated state by PTP activity. UV activation of EGFR results from oxidative inhibition of PTP activity.

## 633

### Identification of a Role for Mitochondria in Transcription Factor Activation and Gene Regulation

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Transcriptional expression of genes in normal human epidermal KC by ultraviolet (UV) A radiation was previously shown to result from the generation of ceramide from cell membrane sphingomyelin. Ceramide then serves as second messenger to activate transcription factor AP-2 which mediates increased transcriptional expression of UVA radiation-inducible genes such as ICAM-1. In the present study we analyzed the mechanism by which ceramide activates AP-2. We were particularly interested in the role of mitochondria in this system because we have observed that ceramide-induced AP-2 activation and ICAM-1 mRNA expression could be inhibited upon treatment of KC with inhibitors of the mitochondrial (mt) electron transport chain. Ceramide-induced apoptosis is known to involve the release of cytochrome C from the mitochondria through their voltage dependent ion channel (vDAC; = permeability transition pore) into the cytoplasm. We now report that stimulation of KC with C<sub>2</sub>- or C<sub>6</sub>-ceramide at concentrations which did not cause apoptosis but AP-2 activation and ICAM-1 induction, also lead to the release of mt cytochrome C into the cytoplasm of KC (Western blot analysis). Even more important, treatment of ceramide-stimulated KC with substances, which prevent opening of the mt vDAC (e.g. Carboxyatractylolide and Bongkretic acid) inhibited (i) the release of mt cytochrome C into the cytoplasm (ii) AP-2 activation (GEMSA) and (iii) increased ICAM-1 transcription (RT-PCR). This effect was specific because under identical experimental conditions IFN- $\gamma$ -induced ICAM-1 mRNA expression was not prevented. Mitochondrial cytochrome C, after its release into the cytoplasm of KC, might thus have the capacity to activate transcription factor AP-2 and thereby induce gene transcription. In order to test this possibility the AP-2 was coincubated *in vitro* with cytochrome C. Photometric analysis revealed that this interaction was associated with a redox change, i.e. cytochrome C was reduced, whereas AP-2 was oxidized. The latter effect was of functional relevance, because oxidized AP-2 exhibited a greatly increased binding capacity for AP-2 consensus sites (GEMSA). These studies indicate a previously unrecognized role of mitochondria in transcription factor activation and gene regulation in mammalian cells.

## 635

### Ultraviolet-A Irradiation Induces Expansion of Intraepithelial Tumor Cells in a Tissue Model of Early Cancer Progression

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One of the early events in skin cancer associated with ultraviolet-A (UV-A) is thought to occur as clones of damaged keratinocytes escape apoptosis and undergo clonal expansion at the expense of adjacent normal cells. However, factors leading to expansion of potentially malignant cells are not well understood due to a lack of tissue models which mimic these early events. The goal of this present study was to further characterize the early events in UV-induced skin neoplasia. To accomplish this, we generated organotypic cultures which mimic premalignant disease by mixing normal keratinocytes (NHK) with  $\beta$ -galactosidase ( $\beta$ -gal)-marked intraepithelial tumor cells (HaCat-ras, clone II-4), which bear mutations in both p53 alleles. The fate of intraepithelial tumor cells was then followed by immunohistochemical staining for  $\beta$ -gal after UV-A irradiation. The effect of UV-A irradiation on NHK and II-4 cells was characterized by measuring UV-A-induced cellular damage, as determined by the numbers of sunburn cells and apoptotic nuclei (TUNEL-positive cells), as well as proliferation (BrdU incorporation into S-phase nuclei). We found that UV-A (30–60 mJ per cm<sup>2</sup>) induced a dose-sensitive, apoptotic response and suppressed proliferation of pure cultures of NHK cells. Neither apoptosis nor proliferation of II-4 cells was altered. This differential response suggested that II-4 cells were resistant to the effects of UV-A irradiation. When 12:1 and 4:1 mixtures of NHK:II-4 cells were irradiated, a significant increase in intraepithelial tumor cell expansion was seen. We concluded that UV-A irradiation caused a dose-dependent induction of apoptosis in NHK, while II-4 cells escaped apoptosis, maintained proliferation and subsequently gained a selective growth advantage. This study contributes to our understanding of how UV-A induces intraepithelial tumor cell expansion, thereby accelerating progression towards malignancy.

## 632

### Activation of Protein Kinase B/Akt by Singlet Oxygen is Not Dependent on Growth Factor Receptors

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Reactive oxidizing species (ROS) activate pro- and antiapoptotic signaling pathways. The balance between these pathways is often determined by the concentration of the ROS. Singlet oxygen is an ROS that initiates apoptosis in photodynamic therapy and is produced in skin cells in response to UVA radiation. We have investigated the activation of a cell survival signal protein, protein kinase B (PKB; also called Akt), by singlet oxygen and the relationship of PKB activation to singlet oxygen-induced apoptosis. Singlet oxygen was produced by Rose Bengal (RB) photosensitization in NIH3T3 fibroblasts using 514 nm argon ion laser radiation. Singlet oxygen induced PKB phosphorylation in a time and light-dose dependent manner. Phosphorylation of PKB, detected by immunoblotting, was observed at 5 min, maximal at 10 min and detectable until at least 2 h using 300 mJ per cm<sup>2</sup>. As expected, PKB phosphorylation was dependent on activation of PI3-kinase as shown by the ability of wortmannin and LY 29302 to inhibit this response. Importantly, inhibition of PI3-kinase also enhanced singlet oxygen-induced cell death, demonstrating that this survival pathway is functionally significant. To determine whether activation of growth factor receptors was involved in activation of PKB, as shown previously for H<sub>2</sub>O<sub>2</sub>, inhibitors of receptor tyrosine kinases (suramin), PDGFR (AG 1295) and EGFR (AG1478) were tested. None of these inhibitors decreased the singlet oxygen induced PKB phosphorylation even though the same concentrations inhibited PKB phosphorylation by growth factors. In addition, these inhibitors did not enhance singlet oxygen-induced cell death. MEK but not p38 kinase also contributes to activation of PKB kinase since a MEK inhibitor, PD 98059, but not a p38 kinase inhibitor, SB 203580, inhibited PKB phosphorylation. These results indicate that singlet oxygen-induced apoptosis is modulated by concurrent activation of PKB kinase survival pathway but, in contrast to H<sub>2</sub>O<sub>2</sub>, PKB activation by singlet oxygen does not involve growth factor receptor phosphorylation.

## 634

### A Novel Costimulatory Molecule, Dectin-2, Selectively Mediates Ultraviolet Radiation-Induced Tolerance

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Ultraviolet (UV) light abrogates contact hypersensitivity (CHS) responses and induces hapten-specific tolerance. Although this phenomenon has long been appreciated, molecules specifically involved in this pathway still remains to be shown. We have reported previously that dectin-2 (Dec2) is a dendritic cell-specific C-type lectin receptor with costimulatory function. Therefore, we tested the possibility that Dec2 mediates UV-induced local immunosuppression. C3H/HeN mice that were exposed to low doses of UV light over 4 days (d) and hapten-sensitized through radiated-skin area with dinitrofluorobenzene showed profound inhibition of the CHS response, which was completely prevented upon intravenous injection of soluble recombinant Dec2 (sol-Dec2), an antagonist for cognate Dec2, after the last UV exposure. By contrast, injection of sol-Dec2 did not affect the CHS response in animals that were sensitized and challenged but not UV-exposed. UV-treated mice resensitized 14 d after the first challenge displayed hapten-specific tolerance, whereas UV-exposed mice injected with sol-Dec2 before the first sensitization exhibited a vigorous CHS response. Furthermore, mice that were sol-Dec2-injected with no UV exposure produced the intact CHS response. Adoptive transfer of T cells from regional lymph nodes of UV-exposed mice treated with sol-Dec2 had no effect on the CHS response in recipient mice, whereas the transfer of T cells from UV-treated mice inhibited the immune response. These findings indicate that sol-Dec2 can selectively prevent UV-induced tolerance without affecting effector routes of the CHS response, thereby demonstrating the Dec2-dependent pathway involved in generation of immunological tolerance by UV light.

## 636

### Stat3 in Keratinocytes Plays a Crucial Role in Preventing Apoptosis Induced by UVB Irradiation

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Response of mammalian cells to UV irradiation results in induction of a variety of intracellular signalings, which dictate cell apoptosis or survival. We found that Stat3 in mouse keratinocytes was down-regulated by UVB irradiation. Down-regulation of Stat3 mRNA occurred faster than the decline of the protein expression, suggesting that UVB irradiation affects the Stat3 transcription. Furthermore, we found that keratinocyte-specific Stat3-disrupted mice exhibited an increased number of sunburn cells upon UVB irradiation *in vivo*. Also, their keratinocytes showed an increased susceptibility to apoptosis by UVB *in vitro*. Topical application of the naked Stat3 gene onto the skin not only reversed the UVB sensitivity of Stat3-disrupted mice, but also attenuated apoptosis in wild-type mice. These results indicate that Stat3 plays an important role in preventing apoptosis in keratinocytes induced by UVB irradiation.

## 637

**Ultraviolet Irradiation Reduces NF- $\kappa$ B Inhibitor I $\kappa$ B- $\alpha$  Protein Levels Thereby Activating NF- $\kappa$ B and Up-Regulating NF- $\kappa$ B-Regulated Pro-Inflammatory Cytokines in Human Skin *In Vivo***

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Transcription factor NF- $\kappa$ B is a key mediator of inflammation. We have investigated expression and mechanism of activation of NF- $\kappa$ B by UV irradiation in human skin *in vivo*. Non-irradiated human skin expressed transcripts and proteins for all five members of NF- $\kappa$ B family (p65, p50, p52, RelB and C-Rel), and the NF- $\kappa$ B inhibitors I $\kappa$ B- $\alpha$ , I $\kappa$ B- $\beta$ , and I $\kappa$ B- $\epsilon$ . Levels of NF- $\kappa$ B proteins did not change 30 min–8 h post UV (2MED UVB source). In contrast, I $\kappa$ B- $\alpha$  protein was reduced 25% and 33% at 4 and 8 h post UV, respectively ( $p < 0.05$ ,  $n = 6$ ). Increased DNA binding of p65/p50, but not p52, RelB or C-Rel, was detected at 4 h post UV and maximally increased (2.5 fold) at 8 h ( $n = 6$ ), as determined by electrophoretic mobility shift assay (EMSA). Increased nuclear staining of p50 and p65 was detected at 4, 6, and 8 h by immunofluorescence, consistent with activation of NF- $\kappa$ B. I $\kappa$ B- $\alpha$  gene expression, which is specifically induced by NF- $\kappa$ B, was significantly increased at 5 h post UV ( $p < 0.05$ , 2-fold,  $n = 6$ ), as determined by Real-time RT/PCR. NF- $\kappa$ B-regulated pro-inflammatory cytokines TNF- $\alpha$ , IL-1 and IL-6, were also up-regulated at 4 h post UV ( $p < 0.05$ ,  $n = 8–17$ ). The glucocorticoid clobetasol (0.05%) inhibited UV activation of both NF- $\kappa$ B (77%) and AP-1 (78%), while all-*trans* retinoic acid (RA, 0.1%) inhibited AP-1 (74%), but not NF- $\kappa$ B, as demonstrated by EMSA. Clobetasol reduced UV-induced nuclear localization of NF- $\kappa$ B (p50/p65). Pretreatment with clobetasol, but not RA, inhibited UV up-regulation of TNF- $\alpha$  (66%), IL-1 (65%) and IL-6 (47%). In summary, (1) UV induces NF- $\kappa$ B activation by reducing protein levels of the NF- $\kappa$ B inhibitor I $\kappa$ B- $\alpha$  (2) NF- $\kappa$ B is a critical participant in UV-induced expression of pro-inflammatory cytokines, and (3) the modest anti-inflammatory action of RA is consistent with its inability to inhibit NF- $\kappa$ B-mediated cytokine induction in human skin *in vivo*.

## 639

**Ultraviolet B Radiation Generates Platelet-Activating Factor and Novel Oxidatively Modified Glycerophosphocholines Which act as Platelet-Activating Factor Receptor Agonists in Human Epidermal Cells**

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Ultraviolet B radiation (UVB) has been shown to generate cutaneous inflammation in part through inducing oxidative stress and cytokine production in human keratinocytes. Though the lipid mediator platelet-activating factor (1-alkyl-2-acetyl-glycerophosphocholine; PAF) is synthesized in response to oxidative stress, and keratinocytes express PAF receptors (PAF-R) linked to cytokine biosynthesis, it is not known whether the PAF system is involved in UVB-induced signaling and inflammation. Using select-ion monitoring mass spectrometry, these studies examined whether UVB can generate PAF in epidermal cells. Lipid extracts from UVB treated KB cells contained PAF-R agonistic activity as shown by calcium mobilization assays in both PAF-R-expressing neutrophils and PAF-R-negative KB cells transfected with the PAF-R. Mass spectrometry studies of these lipid fractions revealed that PAF was the major species responsible for the biological activity, but 1-alkyl glycerophosphocholine lipids which contained an sn-2 short-chained 4 carbon modified fatty acid were also detected. Pretreatment with antioxidants inhibited both the biological activity and PAF species generated by UVB. Consistent with the ability of UVB to generate PAF-R agonists, UVB treatment of KB cells expressing the PAF-R resulted in an enhanced epidermal growth factor receptor phosphorylation over control transfected KB cells, which could also be inhibited by antioxidants. These studies indicate that the epidermal PAF-R is a pharmacologic target for UVB through the generation of PAF and novel PAF-like species via oxidative stress.

## 641

**Xeroderma Pigmentosum Variant Heterozygotes Show Reduced Levels of Recovery of Replicative DNA Synthesis in the Presence of Caffeine after Ultraviolet Irradiation**

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Patients with xeroderma pigmentosum variant (XPV) show clinical photosensitivity, skin neoplasias induced by ultraviolet (UV) light, and defective postreplication repair, but normal excision repair. We recently reported an alternative, simple method for the diagnosis of XPV that measures by autoradiography three cellular markers for DNA repair after UV irradiation: unscheduled DNA synthesis (UDS), recovery of RNA synthesis (RRS), and recovery of replicative DNA synthesis (RDS) (Itoh *et al.*, *J Invest Dermatol* 107:349–353, 1996). Among hereditary photosensitive disorders, including other XP, Cockayne syndrome, and UV-sensitive syndrome, only XPV cells exhibited normal UDS, normal RRS, but reduced RDS. This reduction of RDS was enhanced in the presence of a nontoxic level of caffeine. In this study we assess the cellular markers in two independent families that included two photosensitive patients that were identified as XPV. Cells from heterozygote parents showed normal levels of UDS, RRS, and RDS but reduced rates of RDS in the presence of 1 mM caffeine. Furthermore, with a colony-forming assay, the cells showed normal survival by UV without caffeine, but slightly reduced survival by UV with 1 mM caffeine present. In one family, we confirmed inheritance of two heterozygous missense mutations. One mutation is an A to G transition at nucleotide 1840 that generates a K535E missense mutation. Another mutation is an A to C transversion at nucleotide 2003 that generates a K589T missense mutation. These results suggest that XPV heterozygotes can be identified by their sensitivity to UV in the presence of nontoxic levels of caffeine. + Asc-

## 638

**Activation of PI-3-Kinase/Akt Pathway Protects Human Keratinocytes from Ultraviolet Irradiation-Induced Apoptosis**

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PI-3-kinase (PI3K) catalyzes formation of 3-phosphorylated phosphatidylinositols, which in turn activate the serine/threonine kinase AKT. AKT functions to promote cell survival in a variety of cell types. We have investigated the role of the PI3K/AKT pathway in protecting human keratinocytes (KC) from ultraviolet (UV) irradiation-induced apoptosis. UV (2MED from UVB source) increased PI3K activity (2.5 fold,  $n = 3$ ) and activated AKT (5-fold,  $n = 11$ ) within 30 min in human skin *in vivo* and cultured human KC. Pretreatment of KC with the PI3K inhibitors LY 294002 and wortmannin abolished UV-induced AKT activation ( $n = 3$ ), and increased UV-induced apoptosis by 4-fold ( $n = 4$ ), as measured by flow cytometry analysis and DNA fragmentation. UV may induce KC apoptosis by mitochondrial-mediated activation of caspases 9 and 3 and/or FAS-mediated activation of caspase 8. AKT inhibition increased UV-induced release of mitochondrial cytochrome c 5-fold ( $n = 3$ ), and caused appearance of active forms of caspase-9 and caspase-3, 5-fold ( $n = 3$ ). Inhibition of AKT also elevated UV-induced activation of caspase 8 activity 3-fold ( $n = 3$ ). Together these data demonstrate that UV induces apoptosis in human keratinocytes by stimulating mitochondrial release of cytochrome c and with subsequent activation of caspases 9 and 3, and by FAS-mediated activation of caspase-8. UV activation of AKT inhibits cytochrome c release and activation of caspases 3, 8, and 9, thereby protecting KC from UV-induced apoptosis. Selective therapeutic inhibition of AKT in premalignant and transformed KC may be of benefit in the treatment of skin cancer.

## 640

**Apoptosis Induced by Disruption of the Actin Cytoskeleton is Mediated Via Activation of CD95**

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Activation of the death receptor CD95 (Fas/APO-1) either by its ligand or by ultraviolet radiation (UV) is associated with receptor clustering. The mechanism underlying receptor clustering is mostly unclear. There are recent indications that interactions between the cell membrane and the cytoskeleton may play a role in apoptosis. Therefore, we were interested which impact disruption of the cytoskeleton might have on receptor clustering and apoptosis induced by UV. Disruption of the actin cytoskeleton by cytochalasin B (CyB) induced moderate apoptosis, but enhanced UV-induced apoptosis in HeLa cells. CyB exerted its effect on UV-induced apoptosis independently of UV-mediated DNA damage, since induction of DNA repair by addition of exogenous repair enzymes did not alter the effect of CyB. Inhibition of caspase-8, the upstream caspase in CD95 signaling, blocked the apoptotic effect of CyB and its enhancing effect on UV-induced apoptosis. Confocal laser scanning microscopy revealed that (i) CyB induces CD95 clustering, (ii) enhances UV-induced CD95 clustering, and (iii) CD95 clusters colocalize with disrupted actin filaments, suggesting a link between receptor clustering and actin rearrangement. Disruption of CD95 signaling with a dominant negative mutant of the CD95 signaling protein FADD protected HeLa cells from CyB-induced apoptosis and prevented the enhancing effect on UV-induced cell death. The data indicate that disruption of the cytoskeleton causes apoptosis via activation of CD95, demonstrating that death receptors can also be triggered from inside of the cell. Furthermore, cytoskeleton disruption enhances UV-induced apoptosis, possibly via aiding receptor clustering. Together, these data suggest a link between death receptors and the cytoskeleton and that death receptor clustering may be related to cytoskeleton rearrangement.

## 642

**Nucleotide Excision Repair Genes are Up-Regulated by Low Dose UVB: Evidence of a Photoprotective SOS Response?**

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Nucleotide excision repair (NER) is a major mechanism of defense against the carcinogenic effects of ultraviolet light. Ultraviolet B (UVB) causes sunburn and DNA damage in human skin. NER has been studied extensively and described in detail at the molecular level including identification of many NER-specific proteins and the genes encoding NER proteins. In this study, normal human keratinocytes were exposed to increasing, physiologically relevant doses of UVB, and the effect of this exposure on expression of NER genes was examined. An RNA protection assay was performed to quantify transcripts from NER genes, and a slot blot DNA repair activity assay was used to assess induction of the NER pathway. The activity assay demonstrated that cyclobutane pyrimidine dimers were removed efficiently after exposure to low doses of UVB, but this activity was delayed significantly at higher doses. All NER genes examined, including XPG, DDB1, XPC, XPF, RPAp70, DDB2, hHR23B, XPA, RPAp32, RPAp14, CSB, XPB, TFIIHp44, CSA, CDK7, CycH, TFIIHp34, ERCC1, and XPD, demonstrated a similar trend: UVB induces expression of NER genes at low doses (100J per m<sup>2</sup>), but down-regulates expression at higher doses (600J per m<sup>2</sup>). These data support the notion that NER is induced in cells exposed to low doses of UVB, which may protect damaged keratinocytes from cell death. However, exposure to high doses of UVB down-regulates NER genes and is associated with cell death.



## 643

### Stress-Free Hsp70 Induction by Artemia Extract Modulates p53 and p21 Expression and Enhances Human Cells Protection from UVB

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Prior data have shown that human epidermal cells exposed to thermal shock, synthesize Hsp70, which offers UVB-protection a few hours after. They also show that Hsp70 can inhibit apoptosis and even increase cell survival. Our parallel studies have demonstrated that Artemia extract 3% induces in a stress-free manner Hsp70 in human cells. Based on the above, we were interested in investigating, using different UV doses, the protection from UVB insult offered by this stress-free induction of Hsp70, prior to the stress stimuli, in cultured human fibroblasts and *ex vivo* skin organ culture. Our immunoblotting studies showed that at low UVB doses in Artemia-induced Hsp70 cells, p53 and p21 are up-regulated more than in control cells, which suggests an enhancement of cell cycle arrest and DNA repair pathway. This suggestion was confirmed by comet assay which, to the contrary of control cells, showed no UVB-induced DNA degradation in Artemia-induced Hsp cells. Interestingly, at higher doses of UVB, our results were similar and showed that in Artemia-induced Hsp cells, both p53 and p21 levels were higher than in control cells, and exhibited a significant decrease in cell damage and apoptosis, which suggests strongly the action of the repair pathway rather than the apoptosis pathway, while most of the control cells showed the activation of p53-dependent apoptosis. In order to confirm this, Hsp70 was induced similarly in *ex vivo* skin prior to UVB exposure. Interestingly, these skin samples exhibited a great conservation of structure with rare signs of UV damage (rare occurrence of sunburn cells) in contrast to the control skin that exhibited extended signs of damage. Moreover, Langerhans cells immunostaining showed no sign of UV-induced immunosuppression in the Artemia-induced Hsp70 skin samples. These results propose a very interesting and new way of enhancing cell defense against UV stress, using the stress-free Hsp70 induction by Artemia extract.

## 645

### Ceramides Induce Photoaging-Associated Mitochondrial (mt) Mutation

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It is well established that mtDNA deletions in human dermis reflect photoaging. We have previously demonstrated that the most frequent deletion of mtDNA, i.e. the common deletion (CD) can be induced in human fibroblasts and skin. This induction is mediated by reactive oxygen species and closely associated with a decrease of mt function and induction of matrix metalloproteinases. The underlying pathways by which repetitive UV-exposure leads to these effects remain elusive, however. We now report that increase of intracellular ceramides leads to mtDNA deletions and that ceramide- and UV-effects are directly linked. Normal human fibroblasts were repetitively exposed to sublethal doses of C6-ceramide and N-oleoyl-ethanolamin (NOE), a known inhibitor of ceramide degrading proteins. To determine intracellular ceramide levels, lipid extracts were prepared and analysed for ceramide generation by sequential high performance thin layer chromatography allowing separation and subsequent quantification. Repetitive exposure of cells to NOE lead to an increase of intracellular ceramides, not inducing significant cell death or apoptosis. In parallel, exposure of cells to C6-ceramide and NOE lead to the induction of the CD. To determine whether the ceramide effect is in the same pathway as the UV-mediated CD-induction, we repetitively irradiated cells with UVA alone or in the presence of Fumonisin B1, a known ceramide suppressor. Coincubation of cells with Fumonisin B1 significantly delayed the generation of UVA-induced CD, suggesting that UV-mediated and ceramide effects are indeed in the same pathway. This could be further confirmed by coincubation of NOE stimulated cells with singlet oxygen quencher vitamin E which partially abrogated the induction of the CD similar to UVA. Taken together these results indicate a role of endogenous ceramides in the induction of mtDNA deletions and ultimately in the process of photoaging.

## 647

### Lutein Inhibits UVB Radiation-Induced Tissue Swelling and Suppression of the Induction of Contact Hypersensitivity (CHS) in the Mouse

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Studies have suggested that carotenoids have beneficial effects on human health including protection against cancer, macular degeneration and cardiovascular disease. Also, there is evidence that at least some of the immunosuppressive effects of UVB radiation are mediated by radical oxygen species. Lutein is a plant pigment and antioxidant found in marigolds and other plants, including some foods. This study examined whether lutein could inhibit some of the deleterious effects of UVB radiation. C3H/HeJ mice were fed a standard laboratory diet or the diet supplemented with 0.04% or 0.4% lutein. Two weeks after beginning the lutein diet, the ears of 10 mice in each group were exposed to 2000 J per m<sup>2</sup> of UVB radiation (FS40 sunlamps). The thickness of each ear was measured before and 24h after exposure with a spring-loaded micrometer. Ear swelling in response to the UVB radiation was significantly inhibited ( $p = 0.025$ ) in the 0.4% lutein diet group [ $6.4$  ( $\pm 0.01$  mm)]  $\pm 0.7$  (SEM) for control diet,  $4.8 \pm 0.5$  for 0.04% lutein,  $4.3 \pm 0.4$  for 0.4% lutein]. In another experiment, 20 mice were fed each diet for 2 weeks. Then, the shaved dorsum of each of 10 mice in each group was exposed to 1000 J per m<sup>2</sup> UVB radiation daily for 4 consecutive days. One h after the last exposure, 5 irradiated and 5 nonirradiated mice in each of the diet groups were immunized by topical application of dinitrofluorobenzene (DNFB) to the irradiated site. Seven days later each mouse, along with 5 control irradiated, nonimmunized mice and 5 control nonirradiated, nonimmunized mice, was challenged by application of DNFB to the ears and 24 h ear swelling was assessed. As expected, control diet mice exposed to UVB radiation exhibited a significantly ( $p = 0.015$ ) decreased CHS response. However, mice fed the 0.04% or 0.4% lutein diet failed to exhibit significant suppression [ $6.3$  ( $\pm 0.01$  mm)]  $\pm 0.5$  (SEM) for control diet,  $3.4 \pm 0.4$  for UVB/control diet,  $8.6 \pm 1.1$  for UVB/0.04% lutein,  $6.3 \pm 0.3$  for UVB/0.4% lutein,  $0.6 \pm 0.4$  for the nonimmune control group,  $0.9 \pm 0.4$  for the UVB/nonimmune control group]. These data demonstrate that dietary supplementation with lutein inhibits the cutaneous inflammatory response and the local immunosuppressive response to UVB radiation.

## 644

### Cytokine Release and Alloantigen Presentation Measured after UV Exposure of Human Skin

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A large number of experimental and clinical studies performed in the last two decades have established that ultraviolet (UV) light has suppressive effects on the immune system. At cellular level, there are principally alterations of Langerhans cells (LC), production of immunomodulating cytokines by epidermal cells and infiltration into the epidermis of CD11+ macrophages known to have important immunosuppressive activities. Moreover, it has been recently demonstrated that the production of TNF- $\alpha$  and IL-1 $\beta$  by epidermal cells after UV exposure, stimulate the production by macrophages of a matrix metalloproteinase, MMP-9 (92 kDa gelatinase). In the present study in human volunteers, we measured IL-10 and MMP-9 release as well as alterations of Langerhans cells (LC) number and alloantigen presentation after acute UV exposure to solar simulated radiation. Each of the 10 volunteers received 0, 1, 2 and 3 MED on the unexposed buttock skin. Twelve hours after exposure, suction blisters were raised. From blister roofs, we determined LC density (by flow cytometry) and function (using the MECLR assay). We also extracted IL-10 and MMP-9 as described by J. Peguet-Navarro (*Br J Dermatol* 133:660-661, 1995) and quantified them by ELISA. Each end-point showed a SSR dose-dependent response with decrease (*Pex-vivo* assays used in this study are much less sensitive than the *in vivo* methods measuring the UV effect on immune function. They also evidence that a new sensitive *in vitro* method must be developed.

## 646

### Ultraviolet A (UVA) Radiation-Induced Ceramide Generation in Human Keratinocytes (KC) Involves Nonenzymatic and Enzymatic Mechanisms

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UVA radiation was previously found to be capable of inducing the release of ceramide in human KC. Kinetic analysis revealed that UVA radiation-induced ceramide formation follows a biphasic pattern in these cells with a first and strong (17-fold) maximum 1 h after exposure and a second, smaller increase (4-fold) between 24 and 48 h. Moreover, the early and strong peak in ceramide generation was found to be due to nonenzymatic hydrolysis of cell membrane sphingomyelin. In addition to this nonenzymatic pathway, two additional pathways have been identified for ceramide accumulation: hydrolysis from sphingomyelin through neutral and acid sphingomyelinases (Smase's) and de novo synthesis with serine palmitoyl transferase as the rate limiting enzyme. In the present study the mechanism underlying the second, smaller increase in ceramide formation in UVA-irradiated KC was assessed. Lipid extracts from UVA-irradiated, longterm-cultured normal human KC were analyzed for ceramide generation by sequential high performance thin layer chromatography employing a CAMAG AMD2 device, which allows separation of lipids by polarity through a solvent gradient and subsequently their quantification. We have found that inhibitors of neutral Smase such as DCIC as well as inhibitors of acid Smase including chloroquine and ammonium chloride did neither prevent the first nor the second increase in ceramide formation in UVA-irradiated KC. These substances were biologically active, because in the same experiments, IL-1-induced ceramide generation was completely inhibited. Addition of the serine palmitoyl transferase inhibitor cycloserin and the ceramid synthase inhibitor fumonisin B1, however, completely inhibited the second, but not the first increase in ceramide formation in UVA-irradiated cells. These studies indicate that at later time points UVA radiation increases ceramide formation through de novo synthesis. Accordingly, differential RT-PCR analysis revealed a significant increase in mRNA expression for serine palmitoyltransferase 16 h after UVA irradiation, but not for acid or neutral Smase. Solar UVA radiation thus causes ceramide formation in human KC through both nonenzymatic and enzymatic mechanisms.

## 648

### UVA-Filtering Sunscreen Results in Immune Protection that Exceeds Erythema Protection

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Immune suppression is a harmful effect of UV radiation in humans. Earlier studies focused on UVB-induced suppression, but there is also evidence that UVA contributes to this event. Sunscreens have been reported to prevent this phenomenon to varying degrees but differences in the methodologies prevent accurate comparisons. Regardless, immune protection was universally less than erythema protection (SPF). The purpose of this study is to determine the efficacy of a UVA-filtering sunscreen in preventing UV-induced CH suppression, relative to its SPF. 88 human subjects were randomized into 12 groups. 4 had solar-simulated radiation (SSR) at 0, 0.25, 0.5, and 0.75 MED. 7 had sunscreen-protected SSR at 0, 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 MED multiplied by the individual SPF's, before DNFB sensitization. CH response was measured by the millimeter increase in skin fold thickness (SFT) over 5 graded DNFB challenge sites on the arm, 2 weeks after sensitization. SSR dose-response curves were generated and the immune protection factor (IPF) was calculated using linear regression, Weibull and logistic models. Results demonstrate a nonlinear dose-response relationship between UV dose delivered and mm increase in SFT. The CH response was significantly higher in the sunscreen-treated than in the nontreated groups. Significant suppression was observed at 0.5 MED in the untreated groups, vs. 1.0 MEDxSPF in the sunscreen groups. Regardless of the statistical model used, the calculated IPF (range = 32-54) consistently exceeded the SPF (15.97). Because the data follow a sigmoidal distribution, the Weibull model was chosen which yielded an IPF of 55. This UVA-filtering sunscreen was effective in preventing UV-induced CH suppression at suberythemogenic UV doses. Although calculated IPF's varied with the statistical model used, these results provide the first data demonstrating a sunscreen with an IPF greater than its SPF.

## 649

### Aging and Photoaging Dependent Changes of Enzymic and Nonenzymic Antioxidants in the Epidermis and Dermis of Human Skin *In Vivo*

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This is the first comprehensive study of the changes in major antioxidant enzymes and antioxidant molecules during intrinsic aging and photoaging processes in the epidermis and dermis of human skin *in vivo*. We show that the activities of superoxide dismutase and glutathione peroxidase are not changed during these processes in human skin *in vivo*. Interestingly, the activity of catalase was significantly increased in the epidermis of photoaged (163%) and naturally aged (118%) skin ( $n=9$ ), but it was significantly lower in the dermis of photoaged (67% of the young skin level) and naturally aged (55%) skin, compared with young ( $n=7$ ) skin. The activity of glutathione reductase was significantly higher (121%) in naturally aged epidermis. The concentration of  $\alpha$ -tocopherol was significantly lower in the epidermis of photoaged (56% of young skin level) and aged (61%) skin, but this was not found to be the case in the dermis. Ascorbic acid levels were lower in both epidermis (69% and 61%) and dermis (63% and 70%) of photoaged and naturally aged skin, respectively. Glutathione concentrations were also lower. Uric acid did not show any significant changes. Our results suggest that the components of the antioxidant defense system in human skin are probably regulated in a complex manner during the intrinsic aging and photoaging processes.

## 651

### Replenishment of Sun-Depleted Vitamin E in Human Stratum Corneum by a Liquid Skin Cleansing Product

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Previous studies have shown that solar simulated radiation (SSR) depletes human stratum corneum (SC) vitamin E (vit. E). It is not however, known whether exposure to natural sunlight has a similar effect. Nor is it known if the depleted vit. E can be raised above its normal levels via the use of skin cleansers containing vit. E during daily bathing/shower routine. To determine this, 14 female Caucasians ages 18–55, with light to medium skin pigmentation were enrolled into a study. They underwent 2-week of skin preconditioning with cleansers with no vit. E or vit. E acetate (vit. EA). Subjects had no dietary restriction but were asked to limit their sun exposure. On the day of the study, the forearms were exposed for  $30 \pm 1$  min to natural sunlight ( $0.47$  and  $1.17$  mJ per  $\text{cm}^2$  UV-B and UV-A measured at 310 nm and 360 nm, respectively). Prior to and after sun exposure, forearm skin was washed for 1-min with a skin cleanser containing vit. E and vit. EA, and rinsed immediately. The SC vitamins were extracted and analyzed by HPLC. The results indicated that (1) exposure to sunlight, under conditions that did not induce erythema, reduced baseline skin vit. E from  $31 \pm 30$  to  $16 \pm 17$  pmoles per  $\text{cm}^2$  (mean  $\pm$  SD,  $p=0.05$ ); (2) a single wash with the vit. E skin cleanser raised SC vit. E levels from  $31 \pm 30$  to  $204 \pm 79$  pmoles per  $\text{cm}^2$ ; (3) exposure to sunlight depleted this supplemented vit. E by 45% from  $204 \pm 79$  to  $112 \pm 44$  pmoles per  $\text{cm}^2$ ; and (4) in skin pre-exposed to sun, topical application raised vit. E by approximately 500% above the unexposed levels ( $185 \pm 76$  vs.  $31 \pm 30$  pmoles per  $\text{cm}^2$ ). We provide the first evidence that a brief exposure of human skin to natural sunlight in the North-east US seriously depletes skin's natural vit. E. We further show that washing the skin with the vit. E cleanser before sun exposure compensates for vit. E loss. Moreover, using the skin cleanser after sun exposure replenishes the depleted vit. E to levels that will remain approximately 500% higher than natural, pre-exposed levels.

## 653

### Increasing Stratum Corneum Vitamin E: A Comparison of Topical vs. Oral Supplementation

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The objective of this double-blind study was to assess which mode of delivery, topical or oral, more effectively increases the vitamin E content of the stratum corneum. After a seven-day washout period, twenty, adult, Caucasian females were entered into the IRB-reviewed study. Vitamin E was extracted from blood and stratum with solvents and analyzed by HPLC. The subjects were randomly divided among two treatment groups. One group ingested oral vitamin E tablets (400 IU  $\alpha$ -d-tocopherol) daily. The volar forearms of the other group were washed with an experimental body-wash with and without tocopherol and tocopheryl acetate. After nine working days of treatment, vitamin E in blood and stratum corneum were re-analyzed. Topical treatment with the vitamin E bodywash increased stratum corneum tocopherol from 5 to 267 pmoles per  $\text{cm}^2$  and tocopheryl acetate from 1 to 158 pmoles per  $\text{cm}^2$ . In contrast, oral ingestion of 400 IU of vitamin E ( $18 \times$  Recommended Daily Intake, RDI) increased stratum corneum tocopherol levels to 78 pmoles per  $\text{cm}^2$  without affecting tocopheryl acetate levels. Of the two treatments, only oral ingestion significantly ( $p=0.05$ ) increased plasma tocopherol levels from 24.3 to 45.3  $\mu\text{moles}$  per L. Our results show that stratum corneum tocopherol can be increased by either oral or topical routes. However, topical delivery is superior (4-fold) to systemic delivery. We believe that the stratum corneum is a reservoir for the lipophilic vitamin E.

## 650

### DNA Damage and Melanin Production Induced by Ultraviolet Radiation in Human Skin within Various Racial/Ethnic Groups

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The role of racial/ethnic origin in individual UV sensitivity remains unclear. We are evaluating the usefulness of various biomarker methods to measure UV responses in human skin and to investigate the relationships between DNA damage/repair and melanin formation induced by UV exposure in different racial/ethnic groups and phototypes. This study will involve 110 subjects representing different UV sensitivities within 6 racial/ethnic groups. The MED of each individual was determined to assess their sensitivity to UV 1 day before the experimental protocol. Shave biopsies were taken before exposure, immediately after exposure to 1 MED of UV, and then 1 day and 7 days later. Induction of DNA damage (CPD) is detected in frozen sections by indirect immunofluorescence using a monoclonal antibody specific for CPD and with FITC. Nuclear DNA is counterstained with propidium iodide (PI). DNA damage is expressed as the ratio of the intensities of the FITC and the PI fluorescence. The data show that DNA damage measured by CPD is significant immediately following exposure to UV and is gradually repaired towards the background level. While melanin contents measured by Fontana-Masson staining did not change consistently with UV exposure, melanin production increased when DNA damage was more severe and this tendency became stronger as time passed after UV irradiation. The relationships between DNA damage and melanin content showed significant differences among each group of racial/ethnic origin tested and also among each group of phototypes. Melanin staining showed a good correlation with eumelanin but not with pheomelanin content. Thus, individual racial/ethnic origin as well as skin sensitivity affects responses to UV and might predict the risk of human skin cancer. Future study will evaluate the DNA damage evident, the repair efficiency and also the role of melanin in all other skin types.

## 652

### UVA-340 as an Energy Source, Mimicking Natural Sunlight, Activates the Transcription Factor AP-1 in Cultured Fibroblasts: Evidence for Involvement of Protein Kinase-C

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Ultraviolet radiation (UVR) is known to affect a variety of cellular functions, including gene expression, and a number of signaling pathways have been suggested to mediate these effects, including the participation of AP-1, AP-2, and NF- $\kappa$ B. The divergent results from previous studies could be explained, at least in part, by the source of UVR with different spectral characteristics, as well as the type of cells employed as targets. In this study, we have utilized UVA-340, which closely matches the spectrum of natural sunlight over the range of 295–350 nm, as the source of energy for irradiation of human fibroblasts or mouse NIH-3T3 cells in culture. Combination of electrophoretic mobility shift assays and Northern analyses revealed activation of AP-1, but not NF- $\kappa$ B or AP-2. This effect was dependent on de novo protein synthesis, as judged from incubation experiments with cycloheximide (10  $\mu\text{g}$  per ml). Inhibition studies with H7 and HA1004 suggested participation of PKC, but not PKA, and PDO98059, an inhibitor of mitogen activated protein kinase (MEK-1/2) did not alter the AP-1 activation. Free radical quenchers, sodium azide and N-acetylcysteine, did not affect the AP-1 binding activity. Finally, UVA-340 was shown to enhance transcriptional expression of the type VII collagen gene (COL7A1), which is endogenously expressed in dermal fibroblasts and which contains at least one functional AP-1  $\alpha$ -element within its promoter region. Introduction of point mutations into the AP-1 site of the COL7A1 promoter abolished this activation. Thus, the results indicate a role for AP-1 in mediating the enhanced gene expression by UVR, and collectively suggest that AP-1 is an important mediator of UVR action in fibroblasts.

## 654

### IL-12 Completely Blocks the Secretion of TNF $\alpha$ from Cultured Skin Cells: Implications for Photosensitive Conditions

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IL-12 is considered to act primarily on lymphocytes. One recent study showed that IL-12 blunts the rise in TNF $\alpha$  levels that occur after UV irradiation of mice (Schmitt *et al*, *Immunol* 101:90, 2000). Because serum TNF $\alpha$  comes from both lymphocytic and nonlymphocytic sources, we hypothesized that IL-12 might be an important inhibitor of TNF $\alpha$  secretion from many cell types. We now sought to determine if IL-12 could affect the synergistic stimulation of TNF $\alpha$  secretion by UVB plus IL-1 $\alpha$  (Werth *et al*, *J Invest Dermatol* 113:196, 1999). Human skin fibroblasts (FBs) were treated with UVB (30 mJ per  $\text{cm}^2$ ) or sham (zero mJ per  $\text{cm}^2$ ),  $\pm$  IL-1 $\alpha$  (5 ng per ml),  $\pm$  IL-12 (10 ng per ml). TNF $\alpha$  secretion was undetectable from sham-irradiated cells;  $6.2 \pm 2.4$  pg per ml after IL-1 $\alpha$ ;  $8.3 \pm 0.7$  pg per ml after UVB; and  $34 \pm 0.1$  pg per ml after UVB + IL-1 $\alpha$  (mean  $\pm$  SEM,  $n=3$ ). IL-12 totally blocked TNF $\alpha$  production from these cells, regardless of the presence of UVB or IL-1 $\alpha$ . Similar experiments were performed with keratinocytes (KCs) and also showed total suppression of TNF $\alpha$  with IL-12. TNF $\alpha$  was undetectable from sham-irradiated KCs;  $23.2 \pm 4.4$  pg per ml after UVB; but then undetectable after addition of IL-12 to either sham or UVB-treated cells. Exogenous IL-1 $\alpha$  was not added to KCs since previous experiments show endogenous IL-1 $\alpha$  secretion from KCs is sufficient for UVB-induction of TNF $\alpha$ . To determine the molecular mechanism of the IL-12 effect, we transiently transfected FBs with a construct containing the CAT reporter under the control of a 1.2-kb TNF $\alpha$  promoter fragment. 24 h later the cells were exposed to UVB or sham  $\pm$  IL-1 $\alpha$ ,  $\pm$  IL-12. After 24 h, CAT protein (normalized to  $\beta$ -GAL) was  $8.1 \pm 0.2$  from sham-irradiated cells;  $25 \pm 3.7$  after IL-1 $\alpha$  alone;  $21 \pm 1.1$  after UVB; and  $89 \pm 8.7$  after UVB + IL-1 $\alpha$ , consistent with the strong synergy between UVB and IL-1 $\alpha$ . IL-12 totally blocked any effect of IL-1 $\alpha$  (IL-12 + IL-1 $\alpha$ :  $7.1 \pm 0.7$ ; IL-12 + UVB + IL-1 $\alpha$ :  $19 \pm 2.1$ ), while having no effect on the action of UVB (IL-12 + UVB:  $18 \pm 1.7$ ). These studies indicate that TNF $\alpha$  production by noninflammatory skin cells is strongly inhibited by IL-12, and that the mechanism is mediated in part through inhibition of gene transcription via an element within the first 1.2 kb of the TNF $\alpha$  promoter. Because TNF $\alpha$  is involved in the pathogenesis of photosensitive skin diseases (Werth *et al*, *J Invest Derm* 115:726, 2000), IL-12 may play important physiologic and therapeutic roles.

## 655

### Protection Against UV-Induced Genotoxicity and Immunosuppression by Mineral-Containing Sunscreens: *In Vivo* Evaluation

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Molecular mechanisms in skin photocarcinogenesis has assigned a key role to the sunscreen on p53. On another hand, excessive DNA damages will lead to apoptosis, evidenced by "sunburn cells" (SBCs). Impairment of skin immunity through the alteration of Langerhans cells (LC) seems also to be implicated in cutaneous malignancy. Thus, it can be reasonably assumed that the prevention of both SBCs production, p53 induction and LC alteration will be representative of the quality of the photoprotection beyond the simple prevention of erythema. We have developed specific ultrafine containing-mineral sunscreens (SPF25 and 50+) with high absorbance in UVA, specifically designed for children, and present here their capacity to prevent UV-induced genotoxicity and immunosuppression. Twelve volunteers were irradiated on the buttocks, using a solar simulator and skin biopsies were performed 24 h later. Sunscreens were applied either at 2 mg per cm<sup>2</sup> (COLIPA, SPF determination) or 1 mg per cm<sup>2</sup> (more realistic), and specific UV doses (5, 10 and 50 MED) were chosen in order to simulate different behaviours under the sun. SBCs were characterized by morphological features, altered LC and p53 were revealed by immunohistochemistry. In our experimental conditions, we have shown that the two sunscreens were able to significantly reduced SBCs production and p53 induction and to inhibit LC alteration even for high level exposure. When comparing 1 MED-induced cellular damages without sunscreen (UV dose, 1 MED) vs. 1 MED-induced cellular damages with SPF50+ (UV dose, 50 MED), we observed a significant decrease of both genotoxicity and immunosuppression with the sunscreen. These results emphasize the key role of UVA in sunlight damaging effects, and clearly demonstrate that our SPF50+ sunscreen is not only efficient in preventing erythema but also in reducing cellular damages which may lead to skin carcinogenesis in adulthood.

## 657

### Intrinsic Aging vs. Photoaging: a Comparative Histopathologic, Histometric, and Immunohistochemical Study

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Aging of the skin is a complex biological phenomenon affecting the different constituents of the skin. To compare the effect of intrinsic and extrinsic aging processes, 94 biopsies were collected from sun-protected and sun-exposed skin of healthy volunteers representing decades from the 1st to the 9th. Routine H&E staining was used to evaluate epidermal changes. Immunoperoxidase techniques with antibodies against type I and III collagen and elastin were used to evaluate changes in collagen and elastic fibers. Quantitative evaluations of staining intensity and epidermal thickness employed a computer-assisted image analyzer. Epidermal thickness was found to be constant in both sun-exposed and sun-protected skin in different decades, however, it was significantly greater in sun-exposed skin ( $p = 0.0001$ ). In sun-protected skin type I and III collagen were affected only after the 8th decade while in sun-exposed skin staining intensity decreased from 82.5% and 80.4% in the 1st decade to 53.2% and 44.1% in the 9th decade, respectively ( $p = 0.0004$  and  $0.0008$ ). Both collagens appeared disorganized after the 4th decade. The intensity of elastin in sun-protected skin significantly decreased from 49.2% in the 1st decade to 30.4% in the 9th decade ( $p = 0.05$ ). In sun-exposed skin the intensity gradually increased from 56.5% in the 1st decade to 75.2% in the 9th decade ( $p = 0.001$ ). The accumulated elastin was morphologically abnormal and appeared to occupy the areas of lost collagen. Therefore the aging process, whether intrinsic or extrinsic, has quantitative and qualitative effects on both collagen and elastin.

## 659

### Human *In Vivo* Immune Protection Factors of Sunscreens Containing Chemical UV Filters Measured in UV Dose Response Studies in the Local Contact Hypersensitivity Model

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Experimental studies in animals and humans have resulted in a great controversy on the immunoprotective capacity of sunscreens. Thus, we have performed this randomized study to obtain human *in vivo* immune protection factors (IPFs) in the model of UV-induced local suppression of the induction of contact hypersensitivity (CHS) to 2,4-dinitrochlorobenzene (DNCB). Briefly, 73 male subjects were exposed in a multistage approach study protocol to multiples of their individual minimal erythema dose (MED) produced by an Oriol 1000 W solar simulator, left unsensitized or were sensitized 3 days later with 50  $\mu$ L of 0.0625 DNCB on a nonirradiated or UV-irradiated field on the buttock that was unprotected or protected by sunscreen (2 mg per cm<sup>2</sup>). Three weeks after sensitization the volunteers were challenged with different concentrations of DNCB on their upper inner arm and the CHS response was determined at 48 and 72 h by a clinical semiquantitative score, the lesion's diameter, and skin edema measurement by 20 MHz ultrasound. The statistical study analysis by a 4-parameter logistic model revealed that the 50% immunosuppressive dose ranged from 0.71 to 0.79 MED, depending on the endpoint parameter used for quantification of immune response. There was significant immunoprotection by sunscreens ( $p = 0.05$ – $0.001$ ). The IPF of a sunscreen (sun protection factor, SPF 5.2) containing the UVB filter methylbenzylidene camphor (Eusolex 6300) ranged from 5.0 to 6.0 and the IPF of a broad spectrum sunscreen (SPF 6.2) containing methylbenzylidene camphor and the UVA filter butylmethoxydibenzoylmethane (Parsol 1789) ranged from 8.5 to 10.1. Taken together, these results indicate that (1) chemical sunscreens have immunoprotective capacity in humans (2) there is some correlation of the IPF to SPF, and (3) sunscreens with broad spectrum UVA + B protection may exhibit higher immunoprotective capacity.

## 656

### Vascular Endothelial Growth Factor Up-Regulation after UV is Modulated by a TNF- $\alpha$ Antagonistic Peptide

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Vascular endothelial growth factor (VEGF) is one of the potent keratinocyte (Kc) derived angiogenic factors that is induced by UV irradiation (UVR). To determine UV induced VEGF modulations *in vivo*, the lower back of volunteers was exposed to erythemogenic UVR using a solar simulator. In nonirradiated areas, or in areas that were covered with SPF15 sun-block, VEGF was strongly expressed only in the papillary dermis. In contrast, 72 h after UVR, VEGF was induced in the epidermis. Prior reports suggest that following UVR VEGF mRNA in Kc is induced by tumor necrosis factor (TNF)- $\alpha$ , a cytokine up-regulated by UVR in Kc. We investigated whether blocking TNF- $\alpha$  binding to its receptors would inhibit VEGF synthesis and release. We synthesized a small peptide, KWHIVW (MW 887 Da), that is known to interfere with TNF- $\alpha$  binding to its p55 receptor. Normal human Kc or the Kc-delivered cell line SCC 12 F were sham or UV irradiated (30–50 mJ per cm<sup>2</sup>) in the presence of the peptide (2.7 nM) or diluent alone as control. Within 48 h, VEGF 4.0 kb transcript was substantially induced in UV irradiated cells. In addition, within 96 h VEGF levels in medium conditioned by sham vs. UV irradiated cells were  $3.2 \pm 0.1$  fg per cell vs.  $5.4 \pm 0.2$  fg per cell as measured by ELISA ( $p < 0.05$ , non paired t test), expanding earlier reports. Interestingly, addition of TNF- $\alpha$  blocking peptide decreased VEGF levels in irradiated conditioned medium by half ( $49 \pm 2.5\%$ ). Furthermore, cytosolic VEGF levels in Kc treated with the TNF- $\alpha$  binding peptide were substantially reduced as compared to diluent treated cells as determined by immunohistochemical analysis and confocal laser microscopy. Our findings show that in human epidermis VEGF is upregulated after UVR. Furthermore, UVR-induced VEGF synthesis and release from Kc can be inhibited by a peptide that prevents TNF- $\alpha$  binding to its p55 receptor. TNF- $\alpha$  blocking peptide could be used to treat dermatoses characterized by neo-angiogenesis.

## 658

### Percutaneous Drug Delivery Using Er:YAG Laser

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Transcutaneous drug delivery is a potential pathway to avoid the pain associated with needle injections. Since the stratum corneum has a strong barrier function which prevents the invasion of molecules and limits the potential of topical drug diffusion, our aim was to achieve better penetration of these compounds by using an Er:YAG laser to remove the stratum corneum. The Er:YAG laser at the wavelength of 2.94  $\mu$ m, is strongly absorbed by water within the tissue, and can therefore ablate this tissue painlessly with minimal thermal damage. In this study, irradiation with an Er:YAG laser was performed on the volar side of forearms in our human subjects using varying fluences. To examine the effect of the irradiation, we used *in vivo* confocal microscopy (CM), which enables the imaging of the skin, non-invasively. The Er:YAG laser cause immediate white color change (whitening), indicating partial removal of the stratum corneum without inflammation, which was verified with CM. The lowest fluence for whitening varied between individuals. We examined the effect of EMLA, a topical anesthetic, by a pinprick test and pain rating scale. Anesthesia onset was much more rapid after Er:YAG exposure compared with unexposed control sites. We also applied epinephrine at 0.1 mg per ml to demonstrate its penetration into the dermis, which we could visualize as a color change due to vasoconstriction. This demonstrated rapid uptake and the horizontal distribution of the drug. This study suggests that Er:YAG laser is a promising tool for percutaneous drug delivery.

## 660

### The Influence of UVA and UVB on the Tyrosine Kinase Profile of Normal Human Keratinocytes

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Exposure of human skin to UV induces major changes in the genetic program of the exposed cells leading to immediate and long-term skin changes. Although it can be assumed that UV-induced modifications of signal transduction involving protein tyrosin kinases (PTKs) regulate these processes, details as to the specific changes in PTK expression after UV exposure are unknown. To investigate PTK expression in normal human keratinocytes (HNK) we employed a reversed transcriptase-PCR approach using degenerate primers derived from the conserved catalytic domain of PTKs. PCR products were cloned and PTKs from randomly picked colonies (up to  $n = 90$  per screen) were identified by sequence analysis. PTK profiles of sham-irradiated, UVA (filtered metal halide lamp, 60 J per cm<sup>2</sup>), and UVB (filtered metal halide lamp, 256 mJ per cm<sup>2</sup>) treated HNK were analyzed 7 h after exposure. We identified 14 PTKs including 3 receptor kinases (axl, cak, figfr2) and 11 nonreceptor kinases (abl1, abl2, lck, map4k2, fyn, yes, src, cnk, ptk6, mstr1, jak1). The PTK profile of HNK was characterized by a predominance of abl1 and 2. Differential screening revealed a further induction of abl2 expression by UVA. UVB had no influence on abl2 but predominantly induced the expression of the receptor kinases of the axl-family. Both treatments lead to a down-regulation of src-family kinases (src, fyn, yes). Overall evaluation revealed that UVA predominantly stimulates expression of nonreceptor kinases and inhibits receptor PTKs, while UVB had the opposite effect. The differences reached statistical significance at  $p < 0.0001$  ( $\chi^2$  test). We conclude that regulation of PTK expression is part of genetic program that mediates late effects of UVA and UVB. The observed effects are wavelength dependent and affect PTKs which are involved in the regulation of gene transcription, cell death, and proliferation.

## 661

**The Fluorescence of Human Facial Skin is a Good Marker for Aging and Documents the Effects of Retinol Treatment**

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It has been shown that the fluorescence of skin is a good marker for aging in mice and in humans. Specifically, the ratio of the fluorescence of tryptophan moieties to that of pepsin digestible collagen cross-links for the hairless mouse model and the ratio of tryptophan moieties to that of elastin cross-links for human forearm skin decrease monotonically with age. The goal of the present study was to assess the changes in the fluorescence of facial human skin with aging and the reversal of aging changes with application of topical retinoids. The fluorescence of human subjects in the range of 14–75 years of age, in winter and summer was measured on the cheek of 522 volunteers in 5 different geographical areas. The fluorescence intensity was measured with a fiber based fluorescence spectrophotometer equipped with double monochromators (Model: Skinskan, Spex Ind., Horiba Group, Edison, NJ). In a second study 20 individuals with ages 51–70 years were treated with retinol for six months twice a day in a double blind placebo control protocol. All volunteers used a broad spectrum Spf 15 sunscreen for the duration of the study. The fluorescence of the facial skin was measured at baseline, at three months and at the end of the study. In the first study the fluorescence ratio of the tryptophan moieties to elastin cross-links was found to decrease monotonically with age in all the groups and independently of the season. In the second study the fluorescence ratio of tryptophan moieties to elastin cross links was found to decrease for the placebo treated group and remained constant for the retinol treated group. Thus the ratio of the fluorescence intensity of tryptophan moieties to the fluorescence intensity of elastin cross-links is a marker of aging for human facial skin. Furthermore the “slowing-down” of aging changes induced by retinol (or reversal) can be monitored and documented by the fluorescence ratio of tryptophan to elastin cross-links.

## 663

**Keratinocytes Repair UVB- and UVA-Induced DNA Damage More Efficiently Than Fibroblasts or Melanocytes**

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All somatic cells are equipped with DNA repair pathways, which allow them to cope with DNA damage due to endogenous or exogenous agents. In skin, UV-induced DNA damage is of particular importance. In order to compare the efficiency of DNA repair processes in skin-derived cells, we measured the efficiency of nucleotide excision repair (which processes DNA photoproducts) and base excision repair (which processes UVA-typical oxidative base modifications) in primary cultures of neonatal skin fibroblasts ( $n = 13$ ), keratinocytes ( $n = 4$ ), and melanocytes ( $n = 5$ ), using a host cell reactivation assay. In this assay, the plasmid pCMVluc is damaged *in-vitro*, either with UVB/UV-C to produce pyrimidine dimers, or with singlet oxygen to produce oxidative guanine base modification, and is then transfected into the host cells for damage processing. Expression of plasmid-encoded luciferase after 48 h depends on the host cells' DNA repair capacity. With various amounts of DNA damage in the plasmid, keratinocytes exhibited a higher efficiency ( $p < 0.01$ ) of DNA photoproduct repair than fibroblasts (2.3–4.5-fold) or melanocytes (2.3–6.3-fold). Keratinocytes also repaired oxidative DNA damage more efficiently ( $p < 0.01$ ) than fibroblasts (2.4–5.1-fold) or melanocytes (2.0–3.7-fold). Donor variability was not responsible, because similar differences ( $p < 0.01$ ) were observed upon comparing repair efficiency among the three cell types from a single donor. This difference in repair efficiency did not translate into a reduced acute UVB- or UVA-sensitivity of keratinocytes, as shown by cell proliferation and cell cycle FACS analysis. Our data indicate that keratinocytes, which are located at the “forefront” of the ultraviolet mutagenic attack, are equipped with the most efficient repair of UV-induced DNA damage. The data demonstrate a marked difference in DNA repair capacity among cell types and suggest cells most vulnerable to environmental mutagens may have evolved with enhanced repair capacity.

## 665

**Ultraviolet Radiation Suppresses Hapten-Specific Chronic Contact Hypersensitivity**

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Repeated application of hapten induces site-directed shift to the immediate onset of antigen-specific contact hypersensitivity (CHS), also called chronic CHS. Ultraviolet (UV) light is known to exhibit the capacity to impair the induction of conventional, acute CHS, however, it still remains unclear whether UV has any modulatory impact on chronic CHS. To clarify this issue, C3H/HeJ mice, previously referred to as UV-resistant strain, that were first UV-exposed or left untreated, were sensitized with dinitrofluorobenzene (DNFB). 5 days later, mice were challenged with the right earlobes, followed by repeated DNFB application at every third day, totally 12 times. Subsequently, the immediate ear swelling response was observed in non-UV-exposed mice upon challenge, which was significantly prevented with prior UV exposure, indicating that UV suppresses chronic CHS. Interestingly, similar suppression of chronic CHS upon UV was not observed in C3H/HeN that were previously referred to as UV-susceptible. Application of an irrelevant hapten, oxazolone, successfully sensitized animals unresponsive to DNFB-specific chronic CHS, indicating the hapten specificity. There is recent evidence that induction of chronic CHS may be mediated by preferential expression of Th2-type cytokines in repeatedly challenged-ears. To examine if UV alters cytokine profiles in local milieu, semiquantitative RT-PCR analysis was performed, demonstrating that similar expression patterns of cytokine mRNA tested. Furthermore, adoptive transfer of immune lymphoid cells from UV-exposed, repeatedly challenged animals revealed successful transfer of the immunosuppressive phenotype, strongly suggesting that UV-mediated suppression of chronic CHS is, at least in part, mediated by induction of suppressor cells. Together, our present study demonstrates for the first time that UV suppresses hapten-specific chronic CHS. This represent a novel immunosuppressive properties, by which UV impairs immune responses.

## 662

**Both Direct Photodeactivation and Oxidative Damage can Contribute to the Seasonal Loss of Catalase Activity in Human Stratum Corneum**

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The enzymes superoxide dismutase (SOD) and catalase contribute to the antioxidant defense mechanisms of the skin by working in concordance to neutralize superoxide via hydrogen peroxide into water. In human skin, a disturbance in the equilibrium between SOD and catalase would result in a local overproduction of hydrogen peroxide, potentially leading to the formation of the extremely reactive hydroxyl radical through reaction with transition metals. Using a sensitive noninvasive method to measure catalase-like activity on tape strippings from the stratum corneum, we have previously shown that chronic sun exposure leads to a progressive loss of this activity in the skin. The loss of enzymatic activity can be induced by ultraviolet A (UVA) in a dose-dependent fashion, whereas exposure to a dose of UVB that causes visual erythema (1 MED) does not affect catalase activity in the stratum corneum. Deactivation of catalase with UVA can be reproduced on an enzyme solution *in vitro*, suggesting that the loss of activity may partly result from direct absorption by the heme group. SOD on the other hand remains fully active in conditions where catalase is completely inactivated. Exposure of the human skin to 0.5 p.p.m. of the environmental pollutant ozone induces a loss of catalase activity, indicating that oxidative damage by itself can reduce catalase activity. We conclude that chronic sun exposure may induce a seasonal variation in the equilibrium between different antioxidant enzymes in the stratum corneum, by a combination of direct photodeactivation and oxidative damage to catalase. This could lead to a gradual accumulation of damage, especially when the skin is additionally exposed to other oxidizing substances like smoke or pollution. It is therefore important to provide long-term protection for this internal defense mechanism.

## 664

**Ultraviolet Radiation-Induced Suppression of Hapten-Specific Chronic Contact Hypersensitivity is Not Restricted to the *tnf* Gene Locus**

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Ultraviolet (UV) radiation is known to induce local immunosuppression in conventional, acute contact hypersensitivity (CHS) in some, but not all strains of mice. It was previously reported that haplotypes of the tumor necrosis factor (*tnf*) gene locus may determine the fate of UV-responsiveness. Indeed, mice possessing *tnf*(d) are uniformly UV-resistant, whereas those possessing *tnf*(k, b, s, q, j, p) are UV-susceptible. Recently, it was reported that repeated application of hapten induces site-directed shift to the immediate onset of antigen-specific CHS, also called chronic CHS. Therefore, we were interested whether UV has any modulatory impact on chronic CHS and, if so, whether UV-susceptibility in chronic CHS is similarly restricted to the *tnf* gene locus. Mouse strains previously reported to be UV-susceptible (C3H/HeN, C57BL/6, C57BL/10) or UV-resistant (C3H/HeJ, Balb/c, DBA/2) were first UV-exposed over 4 days (d), sensitized with dinitrofluorobenzene (DNFB) through shaved abdomen. 5 d later, the right earlobes were challenged with DNFB, followed by repeated DNFB application at every third days, totally 12 times. As control experiments, the CHS response was monitored after the 1st challenge, confirming the previous observation that UV-induced suppression of acute CHS was only observed in mice previously known to be UV-susceptible. As a consequence of the repeated challenge, the immediate ear swelling response was observed in all non-UV-exposed mice at the repeatedly challenged right earlobes. However, suppression of the response by UV was various. Significant suppression of chronic CHS was observed in C3H/HeJ and DBA/2, while no detectable suppression was appreciated in C3H/HeN, C57BL/6 and C57BL/10. In addition, the suppression was only marginal in Balb/c. Together, the present study demonstrates that UV may suppress hapten-specific chronic CHS, which is not necessarily restricted to haplotypes of the *tnf* gene locus.

## 666

**Investigation of p53 Mutation Through the Analysis of Exons 2–11 in Squamous Cell Carcinoma from PUVA-treated Skin**

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Although PUVA-treated patients are at an increased risk for developing skin cancer, the etiology of and molecular basis for the increased incidence of squamous cell carcinoma (SCC) in PUVA-treated patients are unknown. One of the plausible explanations involves mutations in p53 due to its role in DNA repair and apoptosis. P53 tumor suppressor gene is a major target for carcinogen-specific mutations as revealed in many cancers, and as such, it may be possible to use this gene as a molecular marker to identify the etiology of skin cancers in PUVA-treated patients. The p53 gene consists of 11 exons, among which exons 2–11 code for the protein of 393 amino acids. Early studies noted that most p53 mutations occurred in the regions of the gene which are highly conserved through evolution and presumably of functional importance, primarily in exons 5–8. Thus, most investigators have confined their analyses to these exons. Because of this bias in searching, most of the mutations have been reported in exons 5–8 and their intervening introns. However, from nonskin cancer studies, there is accumulating evidence of mutations in other exons. In this study, we sequenced exons 2–11 from the human skin SCC tissues. After p53 immunohistochemical staining of 9  $\mu$ m paraffin-embedded sections, tumor cells were micro-dissected from hematoxylin-eosin-stained sections without contamination of normal cells. We performed DNA extraction, followed by nested PCR. After the purification of PCR products or the gel extraction of electrophoresed agarose gel, DNA sequencing processes were done. In addition to the new mutation of 723C>T in codon 241 of exon 7, 279G>A mutation was found in codon 92 of exon 4. Both of these were characteristic of solar damage to DNA. This result emphasizes the importance of the analysis of all exons to avoid the potential underestimating of p53 mutation.

## 667

**Avocado Phytosterols Decrease UVB Induced Proinflammatory Mediators**

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Exposure of skin to UVB radiation can cause the production of proinflammatory cytokines and prostanooids. Changes in the production of these mediators in the skin can decrease or increase the potentially damaging effects of UVB irradiation. In this series of experiments we investigated the effects of avocado-derived sterols on the production of pro-inflammatory mediators IL-1 $\alpha$ , PGE<sub>2</sub>, and IL-8 caused by UVB exposure. A thin film of the avocado lotion, or lotion base was applied to EpiDerm 24H prior to UVB exposure, and remained on the tissue for a maximum of 72H. At 12, 24, and 48H postexposure, cells were sacrificed to determine viability, cytokine and prostaglandin production. Prior to UVB exposure treated and nontreated EpiDerm produced over 40 pg per ml IL-1 $\alpha$ . At 12H post UVB exposure there was a complete suppression of IL-1 $\alpha$ . By 48H post UVB exposure, there was a significant difference in the IL-1 $\alpha$  levels in EpiDerm pretreated with the avocado lotion vs. those treated with either the lotion base or UVB alone (5 vs. 13 or 15 pg per ml, respectively). The production of PGE<sub>2</sub> was suppressed for both lotions at 12H. At 48H, there was no significant difference in the production of PGE<sub>2</sub> (~250 pg per ml). IL-8 production was not completely suppressed 12H after UVB exposure. Twenty-four and 48H after exposure, the avocado lotion had a profound effect on IL-8 production compared to the lotion base or UVB alone (468 vs. 1242 or 1464 pg per ml, respectively). Interestingly, 48H after UVB exposure, IL-8 production by untreated control tissue and tissue treated with avocado lotion plus UVB was not significantly different. These data suggest that the addition of avocado-derived sterols to lotions may have long-term effects on chemokines produced by UVB induced inflammation. It is probable that avocado-derived sterols could be used to combat the severity of chronic inflammatory conditions such as atopic dermatitis and psoriasis by down regulating chemokine production, specifically IL-8, by damaged keratinocytes.

## 669

**Topical Retinoic Acid Increases the Proteoglycan Decorin and Elastic Proteins Fibrillin and Tropoelastin in Photodamaged Human Skin**

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Retinoic acid (RA) is the current benchmark therapy for the topical noninvasive treatment of cutaneous photodamage. Topical application RA has been shown to increase the levels of procollagen I and fibrillin in the dermis of photodamaged skin. (Griffiths *et al*, *N Engl J Med* 329:530, 1993; Watson *J et al*, *Invest Derm* 112:782, 1999). We conducted 3, 14 and 28 week clinical trials in healthy volunteers in order to determine what other extracellular proteins were induced by topical retinoic acid. 0.05% retinoic acid was applied daily, for 14 and 28 weeks, to the photodamaged volar forearm of otherwise healthy volunteers. Immunohistochemical analysis of skin biopsies showed that compared to the placebo, 0.05% retinoic acid consistently increased the extracellular proteins decorin, fibrillin and tropoelastin; at 14 weeks the levels of decorin increased in the dermis in 12/15 of the subjects and at 28 weeks in 15/15 subjects. Fibrillin expression increased in 10/16 subjects at 14 weeks and in 12/15 at 28 weeks. Tropoelastin expression increased in 12/16 after 14 weeks of RA treatment and in 13/15 in the 28-week study. The increase in all three proteins occurred principally in the papillary dermis in a region next to the epidermal-dermal junction often referred to as the Grentz- or repair -zone. The stimulation of procollagen I by RA was not as consistent, this protein increased in 9/16 and 10/15 subjects at 14 and 28 weeks, respectively. In a 3-week RA clinical trial following a repeat patch protocol there was no overall increase in the expression of either decorin, fibrillin or procollagen I. This suggests that these ECM proteins are late markers of photodamage repair in skin. To our knowledge this is the first report of enhanced decorin staining in the dermis of retinoic acid treated photodamaged skin.

## 671

**UVB Down-Regulates Desmoplakin in Human Keratinocytes *In Vitro* and *In Vivo***

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Desmosomes are specialized domains of the plasma membrane that play fundamental role in cell adhesion, as receptor molecules and are involved in cell movement during embryogenesis and cancer metastasis. The desmosomal plaque protein desmoplakin (Dsp) is regulated by a variety of signalling pathways, including protein kinase C. Since protein kinase C can be activated by UVB and UVA, we hypothesized that the expression of Dsp in human keratinocytes is influenced by acute UVB and UVA irradiation. To assess this, HaCaT cells were UVB exposed either to single doses of 5, 15, 30 mJ per cm<sup>2</sup> or, on 4 consecutive days, to 5 and 15 mJ per cm<sup>2</sup> UVB, respectively. After 2, 4, 8, and 16 h, cells were immunostained using single and double labeling immunofluorescence and analysed by densitometric image analysis. After both single and repeated exposures to UVB, a time and dose dependent down-regulation of Dsp was observed, exhibiting peaks after 4 and 8 h, at doses starting at 15 mJ per cm<sup>2</sup> for single exposures and 5 mJ per cm<sup>2</sup> for repeated exposures. In contrast, vinculin expression, used as internal control, remained unchanged. Next, to verify whether UV-induced down-regulation of Dsp occurs also in human skin *in vivo*, frozen sections from repeatedly solar simulated UV irradiated buttock skin (suberythemogenic doses on 10 consecutive days) and non irradiated contralateral control sites from healthy young volunteers (n = 8) were immunostained for Dsp. In control skin, Dsp was localized regularly in the lower stratum granulosum and upper stratum spinosum. In contrast, in irradiated skin sites we found a significantly weaker expression of Dsp in the overall epidermis. In summary, down-regulation of Dsp in human keratinocytes is demonstrated *in vitro* and *in vivo*. Further studies are presently undertaken evaluating whether, besides UVB, UVA alone is able to down-regulate Dsp in keratinocytes and if the responsible signalling pathways involve protein kinase C.

## 668

**A Keratinocyte-Specific Pathway Activates NFkB in Response to UV Light**

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Epidermal keratinocytes are natural target cells for UV irradiation and upon exposure activate NFkB, a Rel-family transcription factor. NFkB activation serves to initiate cutaneous inflammation as well as to minimize the cell damage in irradiated keratinocytes. NFkB is activated by phosphorylation of its inhibitor, I $\kappa$ B. While the activation of NFkB by TNF $\alpha$  and other cytokines proceeds via IKK kinase complex, we have previously reported that the activation of NFkB by UV light in keratinocytes does not involve IKKs. Evidence from several studies in HeLa cells suggests that UV can activate NFkB via a Ras-Raf dependent pathway, involving MEK kinase. In present study we have used a battery of inhibitors of this pathway as well as antisense oligonucleotide approaches to compare the UV-responsive NFkB activating mechanisms in HeLa cells and in keratinocytes. Using PD098059 and U0126, we found that in HeLa cells UV light indeed activates NFkB via MEK, an important kinase on Ras-Raf dependent pathway. However, in keratinocytes this pathway does not activate NFkB. We confirmed these results using oligonucleotides targeted against MEK. Therefore, in keratinocytes neither of the known pathways responds to UV light to activate NFkB. Thus we conclude that UV light uses a novel pathway, independent of Ras-Raf and MEK as well as of IKK, to activate NFkB in human epidermal keratinocytes.

## 670

**Chronic and Acute Photodamage are Associated with Accumulation of Protein Oxidation in Human Skin**

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Chronic UV-exposure induces solar elastosis as one of the major characteristics of photoaging, whereas erythema, hyperproliferation and desquamation are observed upon acute UV-irradiation. While there is increasing evidence for the generation of reactive oxygen species (ROS) in skin upon UV exposures, little is known about their pathophysiological relevance in human skin *in vivo*. We hypothesized, that chronic and acute photodamage is mediated, at least in part, by oxidative protein modifications. Biopsies were taken from patients with histologically confirmed solar elastosis (n = 10), and from non-UV-exposed sites of age-matched controls (n = 6) and young healthy subjects (< 0.05). The epidermis and stratum corneum of all subjects revealed no or weak staining intensity. Interestingly, in acute UV-exposed skin there was a significantly higher level of oxidative modification in the epidermis, especially within the stratum corneum. Additionally, morphological changes were observed including hyperkeratosis and parakeratosis. In conclusion, the correlation between solar elastosis and protein oxidation was demonstrated for the first time, which hence may be a relevant pathophysiological factor in photoaging. Furthermore, we present conclusive evidence for an involvement of oxidative stress in barrier perturbation following acute UV-exposure.

## 672

**UVB and H<sub>2</sub>O<sub>2</sub> Induce Expression of PAR, a Marker of DNA Repair, in Human Keratinocytes by Different Pathways**

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Apoptosis is an active form of cell death and is initiated by a variety of stimuli, including reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radicals, and superoxide anion radicals. Previously, it has been shown that UVB irradiation of keratinocytes leads to intracellular production of hydrogen peroxide and that antioxidants can inhibit ROS induced apoptosis. Poly(ADP-ribose) (PAR), a marker of apoptosis, is formed upon activation of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP). We hypothesized, that PAR is formed upon UVB exposure of human keratinocytes in a redox-regulated manner. To assess this, human immortalized keratinocytes (HaCaT) were UVB irradiated and thereafter PAR formation was measured by indirect immunofluorescence using a monoclonal antibody against PAR. Cells were harvested at 4 h, 8 h, 16 h and 24 h after single exposures to UVB doses of 0, 10, 20 and 30 mJ per cm<sup>2</sup>. UVB irradiation induced PAR expression in a dose- and time-dependent manner and clearly preceded formation of DNA laddering. Similarly, it was demonstrated that hydrogen peroxide induced PAR formation in a dose-dependent fashion. However, while hydrogen peroxide induced PAR formation was dose-dependently inhibited by pretreatment with catalase, UVB induced PAR formation was not significantly reduced by pre or postexposure incubations with catalase. Similarly, pre or postexposure treatments with other antioxidants, including vitamin E, superoxide dismutase, and N-acetyl-cysteine did not alter UVB induced formation of PAR. In conclusion, our results demonstrate that both UVB and hydrogen peroxide are capable of inducing PAR formation at very low doses. However, while PAR formation was demonstrated to be redox sensitive in the hydrogen peroxide system, redox regulation does not appear to be responsible for UVB induced generation of PAR.

## 673

**UV-Induced Depletion of Mouse Epidermal Vitamin A is Not Mediated by Oxidative Stress**

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We previously showed that, in hairless mice, epidermal vitamin A (free (retinol) and esterified (retinyl esters)) from hairless mice was strongly decreased following an acute UVB exposure (*Dermatology* 199:302–307, 1999), and that a pretreatment with topical natural retinoids (retinol, retinal and retinoic acid) prevented a UVB-induced epidermal hypovitaminosis A (*Photochem Photobiol*, in press). Here, using the same mouse model, we studied the effects of UVA on epidermal vitamin A content and lipid peroxidation, as well as the putative prevention of both by topical antioxidants. An acute exposure to UVA (Philips Cleo Performance lamps) completely depleted epidermal vitamin A with EC<sub>50</sub>s of 0.25 and 0.5 J per cm<sup>2</sup> for retinyl esters and retinol, respectively; these values were 0.1 J per cm<sup>2</sup> for both retinoids with UVB. A single UVA exposure induced a dose-dependent epidermal lipid peroxidation (EC<sub>50</sub> = 3.5 J per cm<sup>2</sup>) giving rise to 55.4 ± 4.2 nmol LPO per g at 20 J per cm<sup>2</sup>, while UVB, up to 2 J per cm<sup>2</sup>, did not increase the basal concentration of 6.7 ± 0.9 nmol LPO per g. A pretreatment with antioxidants (α-tocopherol, ascorbic acid) did not inhibit UV-induced vitamin A depletion, but inhibited by 96% the increased lipid peroxidation. Thus acute UVA induced both epidermal vitamin A depletion and lipid peroxidation, UVB induced only vitamin A depletion, and topical antioxidants prevented only UV(A)-induced lipid peroxidation. This indicates that the depletion of epidermal vitamin A by UV does not depend on UV-induced oxidative stress, as assessed by lipid peroxidation, but is more likely due to a direct interaction between retinoids and UV light.

## 675

**Epidermal Accumulation of Retinyl Esters Inhibits UVB-Induced Biological Effects in Mouse Skin**

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Vitamin A (vA) is normally present in the epidermis as retinol (ROL) and retinyl esters (RE). Besides its many biological effects, vA is photosensitive since both ROL and RE have an absorption spectrum with a maximum at 325 nm. Accordingly, we and others have previously shown that UVB exposure depletes both epidermal ROL and RE. The biological implications of this is not known. In the present work we have explored the possibility that RE may provide a protective effect towards UVB. Hairless mice were first topically treated with retinaldehyde in order to increase epidermal RE content, and then exposed to 1 J per cm<sup>2</sup> UVB. Topical treatment promoted an increase of basal epidermal RE from 1.2 ± 0.2 to 21.4 ± 0.6 nmol per g (n = 4). In untreated mice, UVB induced, 24 h after irradiation, an increase of epidermal TUNEL positive cells from 0 to 76 ± 30 per mm (n = 8), as well as a 3.7-fold increase of cJun mRNA expression (n = 8). In mice loaded with high RE levels and then exposed to UVB, epidermal RE levels returned to 2.4 ± 0.4 nmol per g, while UVB-induced apoptotic cells were decreased by 70% and increase of cJun mRNA expression was abolished. In order to explore the photoprotective effect of RE, liposomes prepared from RE-enriched phospholipids were loaded with the hydrophilic fluorescent marker indo-1. Such liposomes whose membranes contained 15 mM RE inhibited 99% of the fluorescence emitted by indo-1 and decreased by 59% the UVB-induced bleaching of indo-1. Altogether, these observations show that RE were photoprotective in an *in vitro* model, and when loading the epidermis with high levels of RE, biological effects induced by UVB such as apoptosis and induction of cJun mRNA were significantly inhibited. This indicates that RE, besides their function as vA precursors, have a direct biological action as photoprotectors.

## 677

**Analysis of Cellular Retinoic Acid Binding Protein II after 1, 3, 14 and 28 Weeks Topical Application of Retinoic Acid to Human Skin**

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The topical application of retinoic acid (RA) is the current benchmark therapy for the noninvasive treatment of cutaneous photodamage. RA selectively induces proteins in skin which are involved in retinoid transport and metabolism. One of these proteins is the cellular retinoic acid binding protein type II (CRABP II) whose mRNA levels are increased in skin treated topically with RA and other retinoids. Thus CRABP II has been used as a marker for retinoid bioactivity in cells. Using a monoclonal antibody, specific for CRABP II, we examined the expression of this protein in the photodamaged skin of healthy human volunteers treated with 0.05% retinoic acid for 14 or 28 weeks. CRABP II expression increased in the epidermis in 12/16 of the volunteers' forearms treated with RA after 14 weeks compared to those treated with placebo. Quantitative image analysis showed that this increase was as much as 4 fold. The expression of CRABP II in RA treated skin formed a gradient across the epidermis with highest expression seen in the granulosum and with no detectable expression in the keratinocyte basal layers. Furthermore the location of CRABP II appeared to be polarised on the dermal side of the cytoplasm in those cells expressing the protein. In a 28-week study 14/15 of the volunteers showed a striking increase of CRABP II in the epidermis of skin treated with RA compared with the placebo. The gradient of CRABP II in the epidermis was less evident at 28 weeks and the distribution of the protein in the cytoplasm of cells was more uniform. There was also evidence of expression of CRABP II in dermal cells. CRABP II was also used as a marker of retinoid activity in short-term human clinical trials. Retinoic acid at 0.01, 0.035 and 0.05%, topically applied to photodamaged forearm under a patch for three weeks, significantly increased the protein levels of CRABP II in the epidermis (p < 0.01, n = 12) compared to the vehicle. This increase in expression of CRABP II did not correlate with the erythema score during the study. Retinoic acid (0.05%) and retinol (0.2%) also significantly induced CRABP II in a one week patch test on photodamaged skin (p < 0.05 n = 16). To our knowledge this is first reported assessment of the CRABP II protein, as a useful marker of retinoid activity, in long and short-term human clinical trials on photodamaged skin.

## 674

**Inhibition of PUVA-Induced Photodamage by the Isoflavone Genistein**

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Psoralen plus UVA (PUVA) therapy has been widely used in the treatment of many dermatological disorders. Accumulating evidence shows that long-term PUVA therapy is associated with an increased risk of squamous cell carcinoma and malignant melanoma. Genistein (5,7,4-trihydroxyisoflavone), a major isoflavone in soybeans and a specific inhibitor of protein tyrosine kinase, has been shown to inhibit ultraviolet B radiation induced skin carcinogenesis in hairless mice. In this study, the effects of topical genistein on PUVA-induced photodamage on mouse skin were examined. In two separate experiments, genistein (5, 10, and 20 micromoles) in a dimethyl sulfoxide/acetone (1:9) solution was applied to SKH-1 female mice one hour post psoralen dosing (10 mg per kg) and one hour prior to ultraviolet A (UVA) irradiation (25 kJ per m<sup>2</sup>). Application of genistein significantly decreased PUVA-induced skin thickening and edema, and greatly diminished cutaneous erythema and ulceration in a dose-dependent manner. Histological examination shows that PUVA treatment of mouse skin induced dramatic inflammatory changes as evidenced by epidermal acanthosis, dermal neutrophilic/lymphocytic infiltration, and prominent hydropic degeneration with extensive dyskeratotic and atypical keratinocytes throughout the epidermis. Topical genistein, however, substantially prevented all PUVA-induced histological changes without noticeable adverse effects. Our study suggests that genistein may work as a potential preventive agent against PUVA-induced photodamage and carcinogenesis.

## 676

**Liposomes as Cellular Membrane Model for the Prediction of both UV-filter Capacity and Photoprotective Effect of Lipophilic Molecules**

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We developed an *in vitro* model to assess the UV filter capacity of lipophilic molecules. Liposomes are used as a model of cellular membranes; the molecules of interest are included into liposome membranes, while the inner aqueous compartment encloses the fluorescent marker indo-1. This model can give 2 types of informations: the UV filter capacity of liposome membranes at 330 nm (excitation of indo-1) can be read out by measuring at 405 nm the fluorescence of indo-1 of a liposome suspension excited (with a low intensity) at 330 nm. The intensity of this fluorescence is decreased by the presence in membranes of a molecule absorbing at the excitation wavelength. The second information is the photo-protective index of enriched liposome membranes assessed by measuring the bleaching of indo-1 following exposure to physiological UVB doses (0–50 mJ per cm<sup>2</sup>). Indo-1 is extracted from liposomes, and assayed by fluorometry. In such a model, the indo-1 fluorescence of normal liposomes decreased by 99% and 92% in liposomes whose membranes were loaded with 15 mM retinyl esters (RE) and 15 mM Parsol MCX (a well known UVB filter used in sunscreens), respectively. An exposure of liposomes to 50 mJ per cm<sup>2</sup> UVB destroyed 67%, 27% and 18% of indo-1 in normal liposomes and those enriched with 15 mM RE and 15 mM Parsol, respectively. Another fluorescent marker, 2',7',-dichlorofluorescein (DCFH), was used as another way to assess the photoprotective index of UVB-absorbing molecules. In this case, DCFH, inside the liposomes, is oxidised by UVB, and the oxidation product, 2',7',-dichlorofluorescein (DCF), is fluorescent; thus DCF fluorescence is maximal in liposomes devoid to UVB absorbing molecules. In liposomes enriched with 15 mM RE and 15 mM Parsol, DCF production induced by 50 mJ per cm<sup>2</sup> UVB decreased by 78% and 71%, respectively, as compared to normal liposomes. In conclusion, this model allows to assess the UV filter capacity, as well as the photoprotective index, of lipophilic molecules in a situation simulating that of a biological environment.

## 678

**Efficacy of 308 nm Excimer Laser for Treatment of Psoriasis: Results of a Multicenter Study**T. Salam, S. Feldman, B. Mellen,\* R. Fitzpatrick,† R. Geronemus,‡ D. Vasily, and W. Morison  
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**Objective:** To demonstrate the efficacy of the 308 nm excimer laser for treatment of psoriasis. **Design:** Multicenter open trial. **Setting:** Four dermatology practices (one university-based and 3 private practices) **Patients:** 30 patients per center with stable mild to moderate plaque-type psoriasis vulgaris. **Interventions:** Patients received 308 nm UV-B doses to affected areas. The initial dose was based on multiples of a predetermined minimal erythema dose (MED). Subsequent doses were based on the response to treatment. Treatments were scheduled twice weekly for a total of 10 treatments. **Main Outcome Measure:** 75% clearing of target plaque. Time-to-clearing was analyzed using Kaplan-Meier methods, accounting for truncated observations. **Results:** 124 patients were enrolled in the study and 80 completed the entire protocol. The most common reason for exiting from the study was non-compliance. Of the patients that met the protocol requirements of 10 treatments or clearing, 72% (66/92) achieved at least 75% clearing in an average of 6.2 treatments. 84% of patients (95% confidence interval, CI=79 to 87%) reached =75% improvement after =10 treatments. 50% of patients (95% CI=35 to 61%) reached =90% improvement after =10 treatments. Common side effects were well tolerated and included erythema, blisters, hyperpigmentation and erosions. **Conclusions:** Monochromatic ultraviolet-B (308 nm) phototherapy with the excimer laser is an effective and safe treatment for psoriasis. It requires fewer patient visits than conventional phototherapy, and unlike other phototherapy treatments, the laser targets only the affected areas of the skin, sparing the surrounding uninvolved skin.

## 679

### Evaluation of UVA-Induced Oxidative Stress and Genotoxicity in Human Melanocytes

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 The carcinogenic events that lead to melanoma seem to be distinct from those that result in basal or squamous cell carcinoma. UVB signature mutations are commonly found in basal and squamous cell carcinoma but are rare in melanoma tumors. This and the findings that UVA radiation (320–400 nm) induces melanoma tumors in two animal models suggest that UVA, rather than UVB (290–320 nm), radiation is mainly involved in the malignant transformation of melanocytes. The popularity of tanning booths and the increased frequency of melanoma despite the use of sunscreens that protect from UVB suggest the possible carcinogenic effects of UVA radiation. We have been investigating the responses of human melanocytes to a single irradiation with doses of UVA ranging between 22 and 100 J per m<sup>2</sup>, with peak emission of the UVA source at 350 and 365 nm. Doses = 29 J per m<sup>2</sup> consistently produced a significant reduction in proliferation, due to arrest of melanocytes in G1-S. Human Melanocytes were less sensitive than keratinocytes or fibroblasts to the cytotoxic effects of UVA. Melanocyte death was only observed after exposure to doses of UVA = 85 J per m<sup>2</sup>, while extensive cell death was observed in keratinocyte and fibroblast cultures beginning at a dose of 44 J per m<sup>2</sup>. This may be partially due to the high constitutive levels of Bcl2 and/or the presence of melanin in melanocytes. Stimulation of tyrosinase activity and increased levels of tyrosinase and tyrosinase-related protein 1 in melanocytes were also induced by doses of UVA = 29 J per m<sup>2</sup>, indicating stimulation of melanogenesis. This dose of UVA induced profound phosphorylation of the MAP kinase p38, and irradiation with 38 J per m<sup>2</sup> UVA resulted in a 13-fold increase in hydrogen peroxide generation. These results clearly show that human melanocytes undergo oxidative stress upon irradiation with relatively mild doses of UVA. The resistance of melanocytes to killing by UVA might result in increased mutation frequency and in tumorigenesis.

## 681

### Activation of the Human Elastin Promoter by Ultraviolet Radiation can be Decreased through the Application of Antioxidants

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 Solar radiation produces reactive oxygen species, which may contribute to the activation of the elastin promoter, ultimately leading to an increase in elastin deposition. To determine whether antioxidants reduce the activation of the human elastin promoter (HEP) through screening or free radical scavenging, UVA340 lamps, simulators of short terrestrial solar wavelengths (295–340 nm) were used to treat rat aorta smooth muscle cells, containing the HEP/chloramphenicol acetyl transferase (CAT) gene construct. Cell cultures were incubated with either an antioxidant cocktail mixture (sodium ascorbate; Trolox, a modified vitamin E derivative; and NAC, n-acetyl l-cysteine) or a solution of EGCG (major component in green tea). The extent of screening was first determined by irradiating cell cultures with the antioxidant cocktail either suspended above cell cultures in quartz dishes or in direct contact with the cells. The suspended antioxidant cocktail reduced the relative CAT activity ~ 50%; a further 10% reduction was observed when the antioxidant cocktail was in direct contact with the cell cultures. Incubation of cells with EGCG did not reveal any significant reduction in relative CAT activity. We further explored the effects of the antioxidant cocktail through incubation prior to irradiation, throughout the irradiation period, and after irradiation have been completed. Though pretreatment and direct contact of antioxidant cocktails throughout the UV irradiation period demonstrated reduced relative CAT activity, 25–40% reduction in CAT activity occurred when antioxidant cocktails were applied immediately after UV irradiation. Contrary to commonly accepted views, our findings suggest that antioxidants perform more as screeners of UVR rather than scavengers of reactive oxygen species.

## 683

### Grading of p53 Protein Accumulation in Normal Sun-Exposed and Nonexposed Type IV Human Skin

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 Sun-exposed human skin is known to have increased expression of the p53 protein when compared to nonexposed sites and this skin often shows p53 mutations. Using immunohistochemistry, we assessed the quantitative differences in p53 expression in clinically normal, sun exposed (face) and nonexposed (abdomen) human Type IV skin from the same 40 individuals. The subjects ranged in age from 6 to 83 years. The pattern of p53 expression was graded using a previously established technique by two independent observers. Previous studies of Type I and II skin have demonstrated that when evaluating p53 staining, a dispersed pattern signifies wild-type p53 and a compact pattern is a site of p53 mutation, i.e. clonal proliferation of cells. The majority of our sun-exposed samples 95% (38/40) show a dispersed pattern of p53 staining and an almost linear correlation between decade (1st to 9th) and p53 expression. In sun-exposed skin from the 1st decade the average score obtained on histological evaluation was 0%, this increased to 51.7% in the 4th decade and 100% in the 9th decade. We attribute the lack of complete linearity primarily to variations in the amount of sun-exposure between individuals of the same decade and also the season when the biopsy was taken. Non sun-exposed skin demonstrated minimal to no staining despite the age studied and comparison of exposed and protected sites from the same subjects demonstrated statistically significant ( $p \leq 0.05$ ) p53 expression from the 3rd decade on in exposed skin. In Type I-II skin the dispersed pattern is considered to be a temporary response to sun damage which usually subsides within 15 days. In Type IV skin the dispersed pattern seems to be cumulative and may represent mutations in p53.

## 680

### Single-Stranded DNA Homologous to the 3' Overhang Telomere Sequence Protects Skin Cells from Oxidative Damage

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 Thymidine dinucleotide (pTT), which is partially homologous to the 3' overhang telomere sequence that is exposed during DNA damage and/or repair, and an 11-base oligonucleotide (11mer) with full telomere homology (pGTTAGGGTTAG), evoke a variety of cellular responses like those seen after UV-induced DNA damage. These responses include accelerated removal of photoproducts, cell cycle arrest and apoptosis. Because UV radiation, especially UVA, produces oxidative stress in cells, we compared the ability of pTT to 11mer in protecting human fibroblasts and squamous carcinoma cells (SCC12F) against oxidative damage. In both cell types, optimal concentrations of pTT (100  $\mu$ M) and 11mer (40  $\mu$ M) induced the UV-responsive mitochondrial superoxide dismutase (SOD2) mRNA levels as compared to cells treated with diluent alone. The 11mer induced SOD2 mRNA by ~100% as early as 8 h, and the induction was sustained through 24 h pTT induction followed a similar time course, but SOD2 was induced only ~30% over control levels. Cells pretreated for 3 days with pTT were then exposed to UVA (10 J per cm<sup>2</sup>, 340–400 nm, Sella Sunlight) and cell yield was determined up to 72 h after irradiation. pTT pretreatment significantly enhanced cell survival by ~30% ( $p < 0.002$ , paired t-test) at 48 and 72 hours after irradiation. Our data show that DNA oligomers homologous to the 3' telomere overhang enhance cellular defence mechanisms against oxidative damage, at least in part by upregulating antioxidant enzyme levels. The data suggest that exposure of the 3' telomere overhang during oxidative damage and/or repair results in elevation of antioxidant enzymes, leading to an adaptive response against further damage. Therefore, oligomers with telomere homology could be used to decrease cellular oxidative damage that occurs during aging and cumulative sun damage.

## 682

### Prevention of UVB Light-Induced Leukocytes Infiltration, Oxidative stress, and Depletion of Class II MHC + Iak + Cells by Green Tea Constituent (-)-Epigallocatechin-3-gallate in C3H/HeN Mouse Skin

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 Exposure of solar ultraviolet (UV) radiation, particularly its UVB (290–320 nm) component, to mammalian skin induces infiltration of leukocytes, oxidative stress and depletion of antigen presenting cells which collectively play an important role in the induction of immunosuppression and photocarcinogenesis. Earlier, we and others have shown that topical treatment with (-)-epigallocatechin-3-gallate (EGCG) the major polyphenol present in green tea prevents UV-induced immunosuppression and photocarcinogenesis in mouse models. To define the chemopreventive mechanism of EGCG, immunohistochemical detection techniques and analytical assays were performed. We found that topical treatment of EGCG (1 mg per cm<sup>2</sup> skin area) to C3H/HeN mice before a single UVB (90 mJ per cm<sup>2</sup>) exposure inhibits UVB-induced infiltration of leukocytes, specifically CD11b+ cell type which are considered to be the major source of reactive oxygen species and tolerance-inducing cell types. Treatment with EGCG before UVB exposure was also found to inhibit UVB-induced myeloperoxidase activity, a marker of tissue infiltration of leukocytes, and depletion in the number of antigen presenting cells in the epidermis when detected as a class II MHC + Iak + cells. Class II MHC + Ia + cells plays an important role in maintaining immune response in the skin, and suppressed immune responses has been shown to be associated with UVB-induced skin cancer risk. Pre-treatment of EGCG was also found to decrease the number of UVB-induced enhancement of H<sub>2</sub>O<sub>2</sub> producing and inducible nitric oxide synthase expressing cells, and H<sub>2</sub>O<sub>2</sub> and NO production both in epidermis and dermis of UVB irradiated skin site. Together, these data suggest that prevention of UVB-induced immunosuppression and photocarcinogenesis by EGCG may be associated with the prevention of UVB-induced infiltrating leukocytes, antigen presenting cell depletion and oxidative stress. A detailed study of possible beneficial effects of green tea in human skin is warranted.

## 684

### Glycated Proteins as Photosensitizers of DNA Damage in Skin Photoaging

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 Chronic exposure to sunlight has been implicated in skin aging and carcinogenesis. The molecular mechanisms of photoaging by UVA, the sunlight's major UV constituent, are poorly understood. DNA molecules are susceptible to genotoxic damage caused by the light absorption of endogenous or xenobiotic photosensitizers. Glycation is a process of accumulative protein damage by reducing sugars and other reactive carbonyl species leading to the intra- and extracellular formation of complex yellow-brown chromophores called advanced glycation endproducts (AGEs). Accumulation of AGEs on dermal elastin and collagen occurs during normal skin aging in humans. Recently, reduced viability of human dermal fibroblasts exposed to UVA irradiation in the presence of protein modified by AGEs has been attributed to the generation of reactive oxygen species. Here we describe the photosensitization of DNA damage by AGE-protein and its inhibition by thiols *in vitro*. Irradiation of covalently closed circular FX-174 DNA with increasing doses of solar simulated light in the presence of AGE-BSA was used to detect photosensitized DNA nicking as a measure of DNA photodamage. The damage was concentration dependent with respect to AGE-BSA as the photosensitizer. Unmodified BSA displayed no such photosensitizing activity. Addition of several antioxidants modulated the photosensitization effect. Mannitol (a hydroxyl radical quencher) and sodium azide (a singlet oxygen quencher) blocked the photosensitized DNA cleavage, whereas catalase and SOD were not effective, indicating the involvement of photoactivated oxygen and hydroxyl radicals. D-Penicillamine (an antioxidant thiol) inhibited the photosensitization effect of AGE-BSA in a dose dependent relationship. Photosensitized DNA damage in skin is thought to be an important mechanism of UVA phototoxicity. Our results suggest that glycated skin proteins can function as photosensitizers of DNA damage and may be important in the genotoxic consequences of skin photoaging.

## 685

***In Vivo* Regulation of Fas and FasL Expression in Human Skin after UVA and UVB Exposure**

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The Fas receptor (CD95/APO-1) can induce apoptosis in human keratinocytes both when stimulated with the ligand FasL and when stimulated directly with UV radiation. Human keratinocytes are known to coexpress both Fas and FasL, and *in vivo* treatment with UVB radiation increases expression of FasL in psoriatic skin. Since apoptosis of keratinocytes induced by UV radiation is an important event in photocarcinogenesis, we wanted to investigate the expression of Fas and FasL in human skin after *in vivo* UVA and UVB irradiation. Healthy volunteers were irradiated with 3 MED UVA (n = 5) or 3 MED UVB (n = 6) on previously unexposed buttock skin. Skin biopsies were taken from unirradiated skin and 12, 24 and 72 h after irradiation. Expression of Fas and FasL was demonstrated by immunohistochemistry on cryostat sections. In all subjects both UVA (n = 5) and UVB (n = 6) irradiation resulted in increased membrane expression of Fas on epidermal cells with maximum expression 12–24 h after irradiation. Expression of FasL on epidermal cells was also increased after UVA irradiation (n = 5) where the distribution was heterogeneously with maximum FasL expression in the stratum spinosum. In contrast UVB irradiation resulted in decreased expression of FasL 12 and 24 h after irradiation, but at 72 h the expression had returned to basis as in unirradiated skin. The role of the dual expression of both CD95 and its natural ligand CD95L on keratinocytes has not been established. Our results show that the expression of both these molecules is influenced by UV radiation. The possible role of this phenomenon in relation to photocarcinogenesis remains to be elucidated.

## 687

**Keratinocytes and Fibroblasts Show Differential Regulation of Human Hyaluronan Synthase-2 Expression During Stimulation with Cytokines and Ultraviolet Irradiation**

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After irradiation, human skin increases its content of hyaluronic acid, a matrix component produced by the hyaluronan synthase (HAS) enzymes. We reported that the major HAS of human skin is HAS-2, and that UV and UV-induced cytokines increase HAS-2 message in human fibroblasts (FBs). Because keratinocytes (KCs) also synthesize HA, we now sought to compare HAS-2 regulation in KCs and FBs. Human skin KCs were irradiated with UVA (5 J per cm<sup>2</sup>), UVB (30 mJ per cm<sup>2</sup>), or sham, and HAS-2 mRNA normalized to 18 s was measured 24 h later. IL-1 $\alpha$  added to sham-irradiated KCs increased HAS-2 mRNA levels by 58%, from  $0.03 \pm 0.01$  to  $0.05 \pm 0.01$  (mean  $\pm$  SEM, n = 3), while FBs exhibited a three-fold increase in HAS-2 expression. UVA increased HAS-2 message in KCs by 94% ( $0.07 \pm 0.02$  without added IL-1 $\alpha$ ;  $0.1 \pm 0.01$  with IL-1 $\alpha$ ), similar to the increase seen with FBs. UVB stimulated HAS-2 message in KCs by 140% ( $0.08 \pm 0.01$  without IL-1 $\alpha$ ;  $0.09 \pm 0.01$  with IL-1 $\alpha$ ), while FBs exhibited a 4.7-fold increase with UVB and 13.3-fold increase with UVB + IL-1 $\alpha$ . We previously found that IL-1 $\alpha$  in combination with UVB, but not UVA, induces TNF $\alpha$  secretion from fibroblasts (Werth *et al.*, *J Invest Dermatol* 113:196–201, 1999), and that addition of anti-TNF $\alpha$  antibodies to FB cells exposed to IL-1 $\alpha$  plus UVB reduced the HAS-2 mRNA level to a value indistinguishable from the effect of IL-1 $\alpha$  alone. We next examined the effect of UV-induced cytokines on HAS-2 expression in KCs. TNF $\alpha$  increased normalized HAS-2 by 51% over sham (from 0.45 to 0.68), and IL-1 $\alpha$  increased HAS-2 by 30% (from 0.49 to 0.58). This is in contrast to the 8.37-fold increase in HAS-2 in TNF $\alpha$ -treated FBs and the 3-fold increase in HAS-2 with IL-1 $\alpha$ . In summary, HAS-2 mRNA is stimulated by UVB and UV-induced cytokines in FBs far more briskly than in KCs. These actions likely contribute to the pattern of increased cutaneous hyaluronan after irradiation *in vivo*, that is, more pronounced in the dermis than in the epidermis.

## 689

**Inhibitory Effects of Niacin in Ultraviolet Irradiation and Reactive Oxygen Species Induced Apoptosis**

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Niacin (NA), a naturally occurring vitamin and a protease inhibitor, was known well to be effective in treating some skin diseases. Previous studies showed that NA had therapeutic effects on several human diseases through inhibiting cell proliferation or inducing cell differentiation. However, the exact biological function of NA on skin cells is still unknown. In the present study, we focused on demonstration of the effects of NA on ultraviolet irradiation (UV) or reactive oxygen species (ROS) mediated cell injury in fibroblast and keratinocyte cell lines. We found that ultraviolet irradiation and hydrogen peroxide treatment were able to cause DNA damage and cell death in fibroblasts and keratinocytes and the cytotoxic effects were associated with elevated amount of intracellular ROS. Addition of low concentrations of NA followed by UV or H<sub>2</sub>O<sub>2</sub> treatment resulted in significant inhibition of apoptosis in a dose and time-dependent manner, accompanied by decreasing the amount of 8-OH-dG measured in fibroblasts and keratinocytes. Nevertheless, the free radical levels were not different statistically between cells with or without niacin pretreatment. These findings suggest that niacin can, at least partially, prevent UV or H<sub>2</sub>O<sub>2</sub> induced apoptosis through reducing DNA damage and enhancing the DNA repair systems.

## 686

**Photobiological and Molecular Mechanisms of UVA-1-Induced Apoptosis in Malignant T-Cells**

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We have previously reported that UVA-1 phototherapy induces apoptosis in skin-infiltrating T-cells and can thus be effectively used to treat patients with cutaneous T-cell lymphoma. In addition, our *in-vitro* studies have revealed that malignant T-cells exhibit a higher susceptibility to UVA-1 radiation-induced apoptosis than normal T-cells. In order to better understand this exquisite sensitivity of malignant T-cells, in the present study the photobiological and molecular mechanisms underlying UVA-1-induced apoptosis were further analyzed in malignant (Jurkat) vs. normal T-cells. UVA-1 radiation-induced apoptosis in malignant T-cells did not involve ceramide-signaling, because addition of exogenous ceramide to malignant cells induced apoptosis at a rate identical to that observed in normal ones. Treatment of malignant T-cells with FAS antibody or FASL-transfected to down-regulate FAS surface expression, however, decreased the susceptibility of Jurkat cells towards UVA1-induced apoptosis to normal levels, indicating the involvement of the FAS system. FAS-induced apoptosis is mediated further downstream by caspase-3. It was therefore of great interest that normal T-cells had significantly lower procaspase-3 levels as compared with malignant T-cells, and that addition of a caspase-3 inhibitor completely prevented UVA-1-induced apoptosis in malignant cells. Finally, an increased sensitivity towards apoptosis was also observed in malignant cells in comparison with normals, when T-cells were left unirradiated but treated with singlet oxygen, which was generated through thermal decomposition of the endoperoxide of NDPO<sub>2</sub>. These findings indicate that upon singlet oxygen-induced activation the Fas signaling pathway determines the susceptibility of a given T cell towards UVA-1 induced apoptosis, possibly at the level of caspase-3 expression.

## 688

***In Situ* Visualization of UV-Induced DNA Damage in Human Fibroblasts Following Microbeam Irradiation**

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In order to examine the interplay of DNA damage and repair proteins *in vivo*, microbeam UV-irradiation (100 J per m<sup>2</sup>) of normal human and xeroderma pigmentosum complementation group A (XP-A) fibroblasts was performed using an isopore polycarbonate membrane filter mask (pore size 3.0  $\mu$ m). We then visualized the induction of both cyclobutane pyrimidine dimers (CPD) and (6–4) photoproducts (6–4PP) using laser scanning confocal microscopy with damage-specific monoclonal antibodies. PCNA, an auxiliary protein for DNA polymerase involved in nucleotide excision repair (NER), was also visualized using anti-PCNA antibodies. We found that CPD and 6–4PP were produced in subnuclear volumes of normal and XP-A cells as several 3  $\mu$ m-foci per nucleus. Normal cells repaired 6–4PP completely within 3 h, and removed half of CPD at 24 h after irradiation. Detergent-insoluble PCNA was not detected without UV but was induced 30 min after irradiation at the subnuclear sites which superimposed on the sites of DNA damage. Fluorescence of PCNA foci decreased with increasing repair times, and became very weak at 24 h. In contrast, XP-A cells could hardly repair both CPD and 6–4PP within 24 h. As expected, almost no PCNA foci appeared within 3 h. However, they were brightly detected at 9 and 24 h at the sites which were overlapped with the damage sites. The present results suggest that DNA photoproducts remain in relatively fixed subnuclear sites during repair, and NER proteins are recruited to their sites for repair. Furthermore, the results suggest that there are two types of PCNA complex formation at the damage sites, stemming from normal NER and from defective NER.

## 690

***In Vivo* Confocal Imaging of Skin Dynamics After 0.5, 1 and 2 MED of UVB Exposure**

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**Introduction:** Near infrared reflectance confocal microscopy (CM) is a technique that allows skin to be imaged *in vivo* with high resolution and contrast. **Objective:** This study was designed to investigate *in vivo* histologic changes after a single exposure to ultraviolet B (UVB) in human skin. **Material and Methods:** Inner forearm skin of 16 adult volunteers was exposed to 0.5, 1 and 2 MED of UVB. Skin sites were imaged from 15 minutes to 3 weeks after UVB exposure by using *in vivo* CM. Biopsies were obtained at various time points from 10 subjects. **Results:** Basal keratinocytes show increased brightness immediately after exposure to 0.5 MED. After 24 hours, highly refractive structures that may correspond to sunburn cells are clearly seen in the malpighian compartment. In the papillary dermis, leukocyte adhesion to the vessel walls and perivascular infiltrate is clearly seen. Thickness of stratum corneum peaks at 5–7 days and reduces thereafter. Stratum granulosum shows focal thinning after 24 hours of 1 and 2 MED and reaches its maximum thickness at 72 hours. It returns to normal after 3 weeks in 0.5 and 1 MED but remains significantly thickened in 2 MED. Epidermal thickness starts to increase at 48 hours and peaks at 5 days, being twice the normal. It continues to reduce thereafter, but does not return to normal even after 3 weeks in any dose of UVB. All these changes correlate well with histology. **Conclusion:** Reflectance confocal microscopy allows a better understanding of skin dynamics after UVB radiation.



## 691

### Quantitative Photographic Method to Assess Suncare Product Spreading

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Sunscreens are used as protection from the harmful effects of the sun. The sun protection factor (SPF) is a measure of the effectiveness of a sunscreen to absorb ultraviolet light. The most important attributes determining SPF efficacy are the UV absorber, amount of applied sunscreen (mg per cm<sup>2</sup>) and quality of the deposited film. SPF's are determined after applying 2 mg per cm<sup>2</sup> of sunscreen; however, people rarely spread such amounts and usually apply 0.5–1 mg per cm<sup>2</sup>. No standard measures of the evenness of the applied film are generally accepted. Thus, the objective of this work was to develop a digital, UV fluorescence photographic method to image the applied sunscreen film and to use new computer software to analyze and quantify its uniformity. Photography was accomplished using modified strobe units with UV band pass filters and a camera with a visible long pass filter. Imaging of untreated human forearm skin yielded images which were "bright" in the green channel due to fluorescence of cross-linked collagen in the dermis. Subsequent application of sunscreen led to appearance of dark zones against a bright background as the sunscreen absorbed the incident UV radiation while it does not attenuate the visible emitted radiation. The fluorescence intensity and homogeneity (gradients) within the applied sunscreen films were studied as a function of applied sunscreen (0.5–2 mg per cm<sup>2</sup>). The results showed a diminution in collagen cross-link fluorescence that correlated ( $r=0.99$ ) with the quantity of sunscreen applied. In addition, sunscreen spreading was more homogeneous with 2 mg per cm<sup>2</sup> than with lower applied amounts. In summary, a simple and rapid fluorescence photographic method has been developed that allows visualization and quantification of sunscreen spreading. This noninvasive *in vivo* technique allows comparison between sunscreen formulations for spreading uniformity, water and sand resistance, and can be combined with human SPF determinations to assess the influence of spreading on the obtained SPF.

## 693

### Retinoic Acid Promotes New Capillary Formation through Growth Factors to Replace the Loss of the Vascular Network in Photodamaged Skin

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We have examined the structure of the capillary network in the papillary dermis in aged photodamaged skin compared to young protected skin. The capillary loop height and the distance from the subepidermal plexus to the outermost viable layer of the stratum granulosum were significantly shorter ( $p < 0.001$ ) in aged photodamaged skin. In several areas along the epidermal-dermal junction the capillary bed appeared flatter in structure compared to the rich looping network observed in young skin. Topically applied retinoic acid improves the appearance of photoaged skin and over time reduces the number of fine lines and wrinkles. Previous studies have reported that these changes are associated with the formation of new dermal blood vessels in photodamaged skin following the treatment of tretinoin. In order to obtain a better understanding of the molecular basis for the formation of these new capillaries, we examined the expression of associated markers in a three month RA clinical trial on photodamaged skin. Histological analysis of biopsies from the trial revealed the presence of patent capillaries and new capillary formation in the upper dermis in skin treated with 0.05% RA compared to vehicle controls ( $n = 10$ ). There was also clear evidence of perivascular cell proliferation and dissemination of fibroblast like cells into the dermis. At the molecular level there was increased protein levels of the growth factors VEGF and bFGF in fibroblasts and endothelial cells. The expression of VEGF was often found in the migrating buds of new vessels. The expression of the bFGF receptor also increased in both endothelial and fibroblasts suggesting that the promotion of new capillary formation may be regulated through an autocrine loop in RA treated photodamaged skin.

## 695

### Protective Effects of Resveratrol Against Short-Term Markers of Photocarcinogenesis in a Mouse Skin Model

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Non-melanoma skin cancer is the most common cancer among humans and solar ultraviolet (UV) radiation, particularly its UVB component (290–320 nm), is its major cause. One way to reduce the occurrence of these cancers is via the use of substances (often antioxidants) termed as "photochemopreventive agents". Resveratrol (*trans*-3,4',5'-trihydroxystilbene), a phytoalexin found in grapes, nuts, fruits and red wine, is a potent antioxidant with strong antiproliferative properties. Resveratrol has been shown to afford protection against chemically induced skin carcinogenesis in CD-1 mice. This study was designed to examine if resveratrol possesses a potential to ameliorate the damages caused by short-term UVB exposure to the skin. The effect of a topical application of resveratrol (25 micro mole/0.2 ml acetone/mouse), 30 min before UVB (180 mJ per cm<sup>2</sup>) exposure to the skin of female SKH-1 hairless mice, was evaluated on bifold-skin thickness, skin edema, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) formation, ornithine decarboxylate (ODC) activity and other short-term markers considered important in photocarcinogenesis. Pre-application of resveratrol was found to result in significant inhibition of UV-mediated increase in bifold-skin thickness (52% inhibition) and skin edema (31% inhibition). As evaluated by immunohistochemistry, preapplication of resveratrol was found to result in a significant decrease in UVB-mediated increased generation of H<sub>2</sub>O<sub>2</sub>. Further, topical application of resveratrol was also found to result in significant inhibition (55%) of UVB-mediated induction of ODC enzyme activity, a well-established marker of tumor promotion. Taken together, our results suggest that resveratrol may afford protection against the damages caused by UVB exposure, and that these protective effects may be mediated via its strong antioxidant properties. Because the markers studied here are regarded as early markers of photocarcinogenesis, our results suggest that resveratrol may be developed as cancer chemopreventive agent against photocarcinogenesis and other adverse effects of UVB exposure. Detailed long-term photocarcinogenesis studies to assess the preventive potential of resveratrol are warranted.

## 692

### Mechanism of UVB-Induced Cyclooxygenase-2 Expression

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Increased production of arachidonic acid and prostaglandin (PG) in the skin are characteristic responses following UVB exposure. Cyclooxygenase (COX), which convert arachidonic acid to PG, includes COX-1 and COX-2. In most tissues, COX-1 is constitutively expressed, whereas COX-2 is highly inducible by a variety of tumor-promoting agents such as TPA and UVB. The important role of COX-2 in photocarcinogenesis was confirmed by studies that showed the reduced photocarcinogenesis in mice by selective COX-2 inhibitor. We studied the mechanisms of COX-2 induction by UVB in keratinocytes and fibroblasts using RT-PCR. A single exposure of normal human keratinocytes or HaCaT cells to UVB induced COX-2 mRNA at the peak at 3–6 h. Increased expression of COX-2 by UVB in HaCaT cells was partially inhibited by tyrosine kinase inhibitor (Herbimycin A, Genestein), EGF receptor tyrosine kinase inhibitor (AG1478), Src tyrosine kinase inhibitor (PP2), p38 MAP kinase inhibitor (SB202190), or PKC/PKA inhibitor (H7). Neutralizing antibodies against TNF RI, IL-1, and EGF did not suppress the COX-2 mRNA activation. Significantly lower doses of UVB induced COX-2 expression in XPA fibroblasts compared with normal fibroblasts. These data indicate that various signaling activated by UVB and UVB-induced DNA damage are involved in the COX-2 induction in a complex manner.

## 694

### Photoprotective, Antioxidant and Anti-Inflammatory Properties of $\alpha$ -Lipoic Acid

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$\alpha$ -Lipoic acid (LA) is a cofactor in mitochondrial enzymatic complexes catalyzing the oxidative decarboxylation of  $\alpha$ -keto-acids (e.g. pyruvic acid) to acetyl-coenzyme A. The antioxidant properties of  $\alpha$ -lipoic acid have been extensively demonstrated during the past 15 years.  $\alpha$ -Lipoic acid is a good free radical scavenger and transition metal chelator. Here,  $\alpha$ -lipoic acid is shown to protect human epidermal equivalents against UV-induced MMP-1 and IL-1 $\alpha$  expression, and their loss of viability. In normal human fibroblasts exposed to cigarette smoke,  $\alpha$ -lipoic acid is found to spare cellular thiols. Furthermore,  $\alpha$ -lipoic acid blocks nitric oxide production in LPS-stimulated murine macrophages and inhibits phytohemagglutinin-induced pro-inflammatory cytokines in peripheral blood lymphocytes. The adhesion of Jurkat T-cells to HaCaT keratinocytes was effectively inhibited by  $\alpha$ -lipoic acid in a dose-dependent manner. However, Repeat Insult Patch Test (RIPT) studies conducted with formulated  $\alpha$ -lipoic reveal sensitization reaction. In conclusion, while  $\alpha$ -lipoic acid affords particularly potent photoprotective, antioxidant and anti-inflammatory properties *in vitro*, its sensitization potential impairs development as a cosmetic ingredient.

## 696

### Costimulation with Ultraviolet B and Polyinosinic-polycytidylic Acid Increase Interferon- $\beta$ Production in Human Dermal Fibroblasts

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Ultraviolet light, particularly in wavelengths of 290–320 nm (UVB), is known to have synergistic action on regulation of cytokine productions in the skin. In this study, we sought to determine whether cotreatment with polyinosinic:polycytidylic acid (poly I:C) which is a synthetic double-stranded (ds) RNA, a strong interferon (IFN) inducer, and UVB induces IFN- $\beta$  production from fibroblasts dramatically. Since virus infection triggers IFN production via dsRNA to induce apoptotic cell death, poly I:C treatment mimics virus infection. Although UVB irradiation alone did not induce IFN- $\beta$  production, combined treatments with poly I:C and UVB induced 2–3 fold higher levels of IFN- $\beta$  than with poly I:C treatment alone. This effect was also seen with mRNA expression. Maximum effect was observed when UVB was irradiated immediately after poly I:C treatment. Since approximately 10% of incident of UVB penetrates to the level of dermal fibroblasts, our results suggest that the augmented IFN- $\beta$  production from UVB exposed fibroblasts which are infected with virus may contribute to the increase of IFN- $\beta$  circulation, potentially inducing apoptotic cell death in viral infected cells and anti viral state in virus uninfected cells.

## 697

**Ascorbate and  $\alpha$ -Tocopherol Decrease in Human Skin During Aging**

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Despite increasing evidence that reactive oxygen species play a role in aging, photoaging, and aging associated skin diseases, data about the antioxidant capacity of skin during the aging process are very scarce. For human studies this may be partially due to technical difficulties in measuring low molecular weight antioxidants in skin. We established a refined methodology for the measurement of low molecular weight antioxidants, which allowed us to look for potential changes in ascorbate,  $\alpha$ -tocopherol and urate skin concentrations during aging. To account for inherent differences in skin layers which reflect predominantly exogenously exposed and nonexposed skin, frozen skin biopsy punches were horizontally dissected into an upper layer of 1.5 mm and a lower one of 1.5 mm thickness. We found a significant difference between the amount of ascorbate and  $\alpha$ -tocopherol present in the upper 1.5 mm skin (epidermis and upper dermis) compared to the lower dermal compartment (lower dermis and parts of subcutis). Ascorbate was seven folds higher in the upper 1.5 mm whereas  $\alpha$ -tocopherol had a 6 fold higher concentration in the lower 1.5 mm skin. Ascorbate showed a significant decrease with increasing age in the upper 1.5 mm ( $p < 0.05$ ) and  $\alpha$ -tocopherol concentration decreased with increasing age in both compartments ( $p < 0.01$  and  $p < 0.05$ ), while urate did not show any correlation with age. A decrease of antioxidants has been described in other organs in humans. The data reported in this study indicate that there is a significant decrease of two major small molecular weight antioxidants in human skin during the aging process. Hence, it seems reasonable to suggest a higher dietary intake of fruits and vegetables for older people as one possibility to increase ascorbate and  $\alpha$ -tocopherol levels.

## 699

**Topical Zinc Sulphate – An Antioxidant for Skin**

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Zinc is a trace mineral required for normal growth, development and function. It is especially abundant in skin, which contains 20% of the body's total. Zinc is an essential element of over 200 metalloenzymes. It affects conformation, stability and activity of these molecules. In recent years, studies have revealed that zinc has an important antioxidant role. In cultured skin fibroblasts, supplemental zinc salts protected cellular DNA and lipids from photodamage induced by UVA and UVB. Oral zinc supplements protected mice against UV immunosuppression to contact allergens. Topical zinc salts protected mice against UVB-induced sunburn cell formation. Although the mechanism of action for the antioxidant effect of zinc salts is unknown, one hypothesis involves induction of metallothionein synthesis. Metallothionein is a family of low molecular weight sulfhydryl proteins with a cysteine content of approximately one-third. Metallothionein is a good antioxidant and indeed is stimulated by UVB radiation, apparently as a protective mechanism against further free radical attacks. In an attempt to develop a topical zinc formulation for antioxidant protection of human skin, we have formulated  $ZnSO_4$  at levels from 1 to 5% in a solution containing 15% L-ascorbic acid and bioflavonoids. Percutaneous absorption of  $Zn^{++}$  at 24 h was tested in Yorkshire pigs. Skin  $Zn^{++}$  levels were maximal at 3%  $ZnSO_4$  concentration and increased from a baseline of 0.115 mg per g skin to 0.902 mg per g. Equimolar  $ZnCl_2$  was also effective, but is known to be more irritating to skin. After 24 h of  $ZnSO_4$  induction, metallothionein-1 increased approximately 3.4-fold, using Northern analysis. These studies reveal that  $ZnSO_4$  can be formulated in a way that permeates skin and induces metallothionein synthesis. Divalent zinc ion-containing formulations may prove promising for providing antioxidant protection to skin. It should be noted that zinc oxide is insoluble and is not a source of zinc ions for skin.

## 701

**Retinoid Dependent Induction of Apoptosis in UVB-Irradiated Human Squamous Cell Carcinoma Cell Line SCC-12 Involves Caspase-3, Caspase-6, and bcl-xS**

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The effects of UVB light and retinoids on the induction of programmed cell death (apoptosis) in skin cancer are not well understood. In previous studies we found that all-*trans*-retinoic acid (atRA) re-induces the UVB-related suppression of apoptosis in the epidermal squamous cell carcinoma cell line SCC-12 (SCC-12). Here we demonstrate which apoptosis mediators are involved in these events. Confluent SCC-12 were irradiated with UV-B light (0.05–20 mJ per  $cm^2$ ), consecutively treated with  $10^{-5}$  M atRA and incubated for 96 h in the dark. Total-DNA and proteins were extracted, and apoptosis was determined by apoptotic-ladder-PCR, and immunoblotting of a variety of apoptosis mediators. In addition, apoptosis was determined by fluorescence staining of annexin-V and caspase-3. Caspase-3 expression was also determined by FACS-analysis. UVB irradiation with 20 mJ per  $cm^2$  led to a significant decrease of the apoptosis rate as compared to nonirradiated SCC-12. However, incubation with atRA after UVB treatment significantly induced apoptosis as compared to atRA treatment of nonirradiated cells, and UV-B exposed cells without atRA-treatment. Here, Cas-3, Casp-6, Casp-8, and bcl-xS were induced, while bcl-xL was suppressed. The induction of apoptosis by atRA in SCC-12 after UVB-radiation suggests that atRA can restore the ability of SCC-12 to induce apoptosis following UVB-damage. This is unexpected since UVB alone suppresses apoptosis in this cell line, which suggests that SCC-12 are not able to react adequately to UV-B-stress and related cell damage. Our results further demonstrate that UVB-induced apoptosis in SCC-12 is regulated different from normal human epidermal keratinocytes. This nonresponsiveness to UVB of SCC may be one feature in the pathogenesis of malignant transformation from epidermal keratinocytes to SCC.

## 698

**Comparative *In Vivo* Pharmacokinetic Study of 8-Methoxypsoralen (8-MOP) Penetration into Human Skin by Microdialysis after Creme, Bath or Oral Delivery**

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Photochemotherapy with oral or topical psoralens combined with long wave ultraviolet light (PUVA) is a standard treatment regimen for various dermatological disorders. However, until now clinical treatment schedules are based largely on phototoxicity testing and determination of 8-MOP serum kinetics while 8-MOP tissue kinetics are still missing. Providing concentration-time profiles with a high temporal resolution microdialysis has been shown to be a suitable method for the characterisation of transdermal drug transport *in vivo*. We used the microdialysis technique to compare tissue concentrations in humans after topical (bath and cream) and oral administration of 8-MOP. The microdialysis is based on a diffusion process through a semipermeable membrane located at the tip of a microdialysis catheter. The catheter is constantly perfused with a physiologic solution (flow rate 0.5–3  $\mu$ L per min). Substances that enter the catheter through diffusion are transported to the outlet tube and can be analysed in the dialysate. Hence, microdialysis allows the sampling and quantification of analytes from the extracellular space of skin. Because proteins can not pass the membrane, it is pharmacodynamically relevant unbound fraction of a substance that can be determined by microdialysis. 8-MOP was applied topical and systemically in 8 healthy volunteers in a cross over study. Dialysate sampling was performed up to 6 h in 20 min intervals. Plasma samples were taken up to 10 h after 8-MOP application. Plasma and tissue concentrations of 8-MOP were determined by LC with double masspectography. Plasma concentrations after oral 8-MOP administration were significantly higher than after topical 8-MOP application. Tissue concentrations were significantly higher and showed fewer variance after topical than after oral 8-MOP. Tissue concentrations decreased fastest after 8-MOP bath. Our data indicate that topical 8-MOP application may be superior to the oral route as skin concentrations are higher and more stable. These data confirm on a pharmacological basis the clinical experience that topical PUVA therapy requires lower cumulative UV-doses compared to oral application.

## 700

**Enhanced Cyclooxygenase-2 Expression in UVB-Induced Murine and Human Skin Tumors**

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Inflammatory stimuli are potent tumor promoters in skin carcinogenesis assays and lead to cutaneous eicosanoid production. Cyclooxygenase (COX), the rate-limiting enzyme for the production of prostaglandins from arachidonic acid, exists in at least two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most tissues and plays various physiological roles whereas, increased COX-2 expression occurs in several types of epithelial neoplasms. Enhanced prostaglandin synthesis has been shown to be a potential contributing factor in UV-induced nonmelanoma skin cancers (NMSCs). Increased COX-2 staining is found in murine skin tumors following chronic exposure to carcinogenic doses of UVB. In this study, using immunohistochemical and Western blot analysis COX-2 expression was assessed longitudinally in a standard mouse UVB complete carcinogenesis protocol and in human basal cell (BCCs) and squamous cell carcinomas (SCCs). Exposure of murine and human skin to UVB caused a transient enhancement of COX-2, which reverted to baseline within hours. Pretreatment with topically applied antioxidants such as green tea extract (1 mg per  $cm^2$ ) largely abrogated the COX-2 response to UVB. However with continuing UVB irradiation of mice, COX-2 persistently increased in hyperplastic skin, benign papillomas and SCCs. Increased COX-2 also occurred in human actinic keratoses and BCCs as well as in murine and human SCCs. The enhanced localization of COX-2 was quite variable occurring in a patchy epidermal distribution in some lesions and limited to the basal cell layer in others. In general, COX-2 expression was more intense in well-differentiated murine SCCs than in benign papillomas. In addition, we observed strong cytoplasmic and perinuclear staining in both murine and human BCCs. Western blot analysis confirmed that normal skin had no detectable signal for COX-2 whereas acute UVB exposure resulted in increased enzyme expression, which continued to increase in developing papillomas and SCCs. In summary, enhanced COX-2 serves as a marker for epidermal UVB exposure and for murine and human NMSCs. These results indicate that COX-2 inhibitors could have potent anticarcinogenic effects in UVB-induced human skin cancer.

## 702

**KGF Exerts Protective Effects on Keratinocytes Following UVB-Induced Cell Injury**

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Keratinocyte growth factor (KGF), a member of the fibroblast growth factor family, has been implicated in wound healing, epidermal proliferation and differentiation. KGF is a paracrine factor produced by mesenchymal-stromal cells and exerts activity on epithelial cells, including keratinocytes. KGF protects hair follicles following cytotoxic injury. More recently, KGF has been shown to act as a mediator of repair in inflammatory bowel disease and chemotherapy-or radiation-induced lung damage. We have previously shown that epidermal growth factor receptor (EGFR) activation protects keratinocytes from cell death induced by UVB. Here, we investigate the potential protective effects of KGF on UVB-mediated injury of human keratinocytes in culture. Treatment with KGF prior to UVB irradiation significantly inhibits loss of clonogenic potential induced by UVB treatment alone. We also show that KGF maintains proliferative potential in UVB-treated keratinocytes. Interestingly, treatment with exogenous EGF prior to UVB irradiation fails to exhibit a protective effect on keratinocyte proliferation or survival. Also, KGF prevents cell death induced by UVB completely, showing similar cell survival as untreated, non-UVB exposed cells. This study provides the first evidence that KGF preserves proliferative potential in human keratinocytes after damage by UVB radiation. KGF also enhances survival of human keratinocytes in culture following UVB-induced damage. Other known growth factors (EGF, TGF- $\beta$ , IL-1 $\beta$ , TNF- $\alpha$ ) failed to elicit this response. These findings bear relevance to clinical dermatology and therapeutics for diseases such as psoriasis, photodermatoses and acute solar injury. KGF and its receptor may play critical roles in cell survival and hyperproliferation, as observed in psoriasis. In photodermatoses and acute solar injury, KGF or pharmacomimetic agents may be of therapeutic benefit in minimizing keratinocyte injury and death.

## 703

### Vitamin C Derivative Ascorbyl Palmitate Promotes UVB-Induced Lipid Peroxidation and Cytotoxicity in Keratinocytes

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Antioxidant application has been proposed as a preventive and protective strategy against the harmful effects of ultraviolet radiation to the skin. A potent antioxidant, ascorbic acid has been shown to protect against sunburn, delay the onset of skin tumors and reduce ultraviolet B (UVB) radiation-induced skin wrinkling. In this study, we sought to determine the antioxidative properties of a lipid-soluble derivative of ascorbic acid, ascorbic acid-6-palmitate (AA6P). We found that AA6P reduced cellular levels of reactive oxygen species following UVB irradiation. Treatment of keratinocytes with AA6P inhibited UVB-mediated activation of epidermal growth factor (EGF) receptor, extracellular regulated kinases (ERK) 1/2 and p38 kinase due to its ability to prevent glutathione depletion and scavenge hydrogen peroxide. However, AA6P strongly promoted UVB-induced lipid peroxidation (LPO), c-Jun N-terminal Kinase (JNK) activation and cytotoxicity. End products of lipid peroxidation such as 4-hydroxy-2-nonenal (4-HNE) have been reported to mediate stress activated protein kinases (SAPKs) activation and cell toxicity in epithelial cells. The lipid component of AA6P likely contributes to the generation of oxidized lipid metabolites that are toxic to epidermal cells. Our data suggests that despite its antioxidant properties, AA6P may intensify skin damage following physiologic doses of ultraviolet radiation.

## 705

### A Novel Nitron-Based Free Radical Trap Blocks UV-Induced Cytokine Production in Human Fibroblasts and Keratinocytes and Inhibits UV-Induced Erythema in Human Skin *In Vivo*

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Exposure of human skin to ultraviolet radiation results in the induction of an inflammatory response which leads to erythema and skin damage. An early effect of UV radiation is the production of IL-1 by keratinocytes and an increase in PGE-2 production in both fibroblasts and keratinocytes. Topical corticosteroids are effective in reducing the production of many of these inflammatory mediators, but long-term use can lead to side-effects, including skin atrophy. Recent studies have shown that oral administration of a proprietary nitron-based free radical trap reduces the production of inflammatory mediators in an animal arthritis model. The aim of this research was to determine if a novel nitron-based free radical trap molecule, CX-412 can block the UV induction of inflammatory mediators in human keratinocyte and fibroblast cell cultures. We also assessed the efficacy of topical formulations of CX-412 in reducing UV-induced erythema in humans. For cell culture studies human fibroblast and keratinocyte cultures were irradiated with 50 mJ of UVB. CX-412 completely blocked the UV light induced increase in PGE-2 in fibroblasts and keratinocytes at concentrations as low as 10 µM. In addition, CX-412 completely prevented the five-fold induction of PGE-2 in fibroblasts treated with 1 ng per ml of IL-1α. Based on these findings, a 2% formulation of CX-412 was used in a double-blind placebo controlled pilot human efficacy study conducted on 12 patients. Patients were irradiated on each forearm with a 3X MED dose of UVB, and either a placebo gel vehicle or the 2% CX-412 formulation applied to the irradiated site. At hourly intervals, patients were evaluated for erythema by both surface spectrophotometry measurements and digital photography. Over 80% of the patients exhibited a marked reduction in visible UV-induced erythema at sites treated with CX-412. Further, the mean difference in erythema between placebo and CX-412 treated sites as determined by surface spectrophotometric measurements was significant (p=0.01). These findings demonstrate that topical formulations of a novel nitron-based free radical spin trap can significantly inhibit UV-induced inflammation and may protect the skin from sun exposure.

## 707

### All-trans Retinoic Acid Promotes the Differentiation of Mouse Neural Crest-Derived Melanocyte Precursors

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Background: Retinoids, a group of chemically related molecules derived from vitamin A, regulate a large number of biological processes in vertebrate development, cell growth, differentiation, and homeostasis. Objectives: We studied the effect of all-trans retinoic acid (ATRA) on the differentiation of melanocyte precursors and the molecular basis of ATRA-induced differentiation. Methods and Results: We have established melanocyte precursors (NCC-melb4) cloned from mouse neural crest cells. NCC-melb4 cells were positive for TRP1, TRP2 and KIT, but negative for tyrosinase and DOPA reaction. They contained stage I melanosomes without those of advanced stages. After 72 hours of ATRA treatment, many of the cells became tyrosinase- and DOPA-positive, changed from polygonal to dendritic in shape, and had stage II-IV melanosomes. RT-PCR analysis showed enhanced expression of tyrosinase mRNA and microphthalmia-associated transcription factor (MITF) mRNA and PKC $\zeta$  mRNA after ATRA treatment. Therefore, it was confirmed that ATRA induced the differentiation of NCC-melb4. The proliferation of NCC-melb4 cells was inhibited by ATRA in a dose-dependent manner. Apoptosis was studied by electron microscopy, TUNEL method and flow cytometry, and was found to have been induced in the cells by ATRA treatment. Electron microscopy showed that the apoptotic cells contained advanced stage melanosomes, suggesting that apoptosis was induced in the differentiated cells. Conclusions: This is the first report to show that ATRA-induced differentiation of melanocyte precursors is accompanied by an increase in MITF mRNA. These results suggest that this transcription factor may be the key molecule in this event.

## 704

### Ultraviolet A Exposure Induces Irradiance-Dependent Vascular Injury in Pig Skin

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Most UV photobiologic responses of skin follow the law of reciprocity; that is, within wide limits the effects are proportional to the sum total of energy delivered (fluence) but independent of the intensity of exposure (irradiance). The present investigation tests whether irradiance is an important variable affecting the responses to UVA (320–400 nm) in a porcine model *in vivo*. A 1000-W filtered xenon arc equipped with a 1-cm-diameter fiber optic was used to deliver UVA to the skin surface of anesthetized white Yorkshire pigs, over a fluence range of 25–150 J per cm<sup>2</sup> and an irradiance range of 40–120 mW per cm<sup>2</sup>. UVA photodamage was quantified histopathologically by a blinded method. UVA exposure caused reproducible alterations that appeared to be primarily vascular in nature. The gross hallmark of this reaction was purpura. Pathologically, by 2 h after exposure to threshold fluences, the superficial dermal vessels exhibited congestion and focal perivascular hemorrhage, with minimal inflammation. By 24 h, leukocytoclastic vasculitis and epidermal apoptosis and/or confluent necrosis occurred, in a strongly irradiance-dependent manner. For example, at 24 h after exposure to 75 J per cm<sup>2</sup>, the width of full-thickness necrosis varied more than 10X over a 2X irradiance range. Likewise, at this fluence the maximal depth of vasculitis varied 4X over the same range. Similar irradiance-dependence was also observed at other fluences tested, with higher irradiances consistently causing more severe effects at a given fluence. These data indicate that photodamage in this model may not be explained entirely by positing the accumulation of a stable photoproduct. Rather, UVA exposure at higher irradiances may dynamically overwhelm the real-time capacity of photoprotective systems such as endogenous antioxidants, leading to the violations of reciprocity observed.

## 706

### *In Vivo* Analysis of Melanocyte Development: Regulation by c-kit and Pax3

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Murine mutations in the growth factor receptor tyrosine kinase c-kit and the paired homeodomain transcription factor Pax3 each result in viable adult mice with ventral white spotting when heterozygous, yet distinct embryonic lethal phenotypes when homozygous. To specify the roles of c-kit and Pax3 in melanocyte development, an *in vivo* analysis of melanocyte development was performed in these mutant backgrounds by intercrossing *Kit<sup>W</sup>/+* (Dominant spotting) and *Pax3<sup>Sp-d</sup>/+* (*Sploch-delayed*) heterozygous mice with mice hemizygous for the *Dct:lacZ* transgene, an *in vivo* marker of melanocyte development. Heterozygous crosses were arranged to generate embryonic litters at E10 – E14.5 of development, and transgenic embryos genotyped and compared for differences in melanoblast presence, location, and number. Results of heterozygous crosses showed that *Kit<sup>W</sup>/Kit<sup>W</sup>* embryos contain fewer melanoblasts than either *+/+* or *Kit<sup>W</sup>/+* embryos from E10 onward. Additionally, cephalic melanoblasts in *Kit<sup>W</sup>/Kit<sup>W</sup>* embryos disappear by E12–12.5. *Kit<sup>W</sup>/+* embryos, though always containing cephalic melanoblasts, have consistently fewer than stage-matched wild-type embryos from E10–14.5. Results of crosses of *Pax3<sup>Sp-d</sup>/+* mice revealed also that melanoblast number at E12.5 is inversely related to gene dosage of the mutant allele, but, in contrast to *Kit<sup>W</sup>/Kit<sup>W</sup>* embryos, *Pax3<sup>Sp-d</sup>/Pax3<sup>Sp-d</sup>* homozygotes feature a limited number of melanoblasts that survive at E12.5. In fact, at E12.5 cephalic melanoblast number in *Pax3<sup>Sp-d</sup>/Pax3<sup>Sp-d</sup>* embryos is comparable to that previously described in *Pax3<sup>Sp</sup>/Pax3<sup>Sp</sup>* embryos, despite the greater effect of the latter allele on other neural crest derivatives. In conclusion, these studies support a role for the receptor tyrosine kinase c-kit from the earliest stages of melanocyte development, days prior to the timepoint of epidermal entry, and indicate the importance of the full complement of receptor for regulating proper proliferation or survival. They also underscore the importance of proper Pax3 gene dosage, and suggest that melanocyte development may be unusually sensitive compared to other neural crest derivatives to the full complement and function of this transcription factor.

## 708

### Fate of Melanocytes During Hair Follicle Morphogenesis

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During hair follicle morphogenesis, melanocyte precursors migrate into developing hair follicles and give rise to differentiated melanocytes that actively produce and transport pigment into keratinocytes of the hair shaft. However, patterns of melanocyte proliferation and differentiation during formation of hair pigmentation unit remain to be elucidated. Using multicolor confocal microscopy and double immunofluorescence of melanogenic proteins [tyrosinase-related proteins 1 and 2 (TRP1/TRP2), tyrosinase] and the proliferative marker Ki67, we show that in C57BL/6 mouse embryonic skin proliferating TRP2+ melanocyte precursors are seen in the hair follicles at stages 1–2 of morphogenesis. In stage 3–4 hair follicles, the majority of intrafollicular melanocytes remain TRP2+ and Ki67+, while some located closely to the forming dermal papilla begin to express TRP1, an early marker of differentiation. First melanin granules appear at stage 5 hair follicles coincident with tyrosinase expression in nonproliferating TRP2+/TRP1+ melanocytes. Stage 7–8 hair follicles actively producing hair show nonproliferating TRP2+ melanocytes in the bulge area, TRP2+/TRP1+ melanocytes in the outer root sheath, and TRP2+/TRP1+/tyrosinase+ melanocytes above the dermal papilla. Taken together, our data show that during formation of the hair pigmentation unit, proliferation of TRP2+ melanocyte precursors is reduced with the onset of expression of differentiation markers TRP1 and tyrosinase, and that melanogenically active melanocytes located above the dermal papilla do not proliferate and express all three melanogenic proteins.

## 709

**Transcriptional Repression Activity and Induction of RPE Neural Differentiation Revealed by Expression of Mash-1 Transcription Factor in Pigment Cell Precursors in Transgenic Mice**

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To understand further the transcriptional determinants of neural crest cell development, the neurogenic transcription factor gene *Mash1* was expressed in transgenic mice using the promoter for the melanogenic gene *dopachrome tautomerase* (*Dti*), expressed early in pigment cell development. *Dti:Mash1* transgenic mice feature coat color dilution and microphthalmia. To analyze neural crest-derived melanoblast development, *Dti:Mash1* transgenic mice were intercrossed with *Dti:lacZ* transgenic mice which permit the *in vivo* assessment of melanocyte development. Results of intercrosses revealed that melanoblasts are reduced in number in E15.5 and E17.5 transgenic embryos, but nonetheless exhibit normal follicular localization. To account for temporal differences in chromogenic substrate reactivity and for the presence of coat color dilution between wild-type and *Dti:Mash1* transgenic mice, co-transfection experiments were performed demonstrating that *Mash1* negatively regulates *Dti* promoter activity and activity from a generic M-box promoter containing nucleotide consensus sequences conserved in the proximal regions of melanogenic gene promoters. In contrast to results with neural crest-derived melanocytes, *Dti:Mash1* transgenic embryos exhibited hyperproliferation of retinal pigment epithelium (RPE) precursors, with induction of expression of neuronal and neuroretinal markers, such as a neuron-specific form of class III  $\beta$ -tubulin and Chx10, that are not normally expressed in developing RPE. In summary, expression of *Mash1* in pigment cell precursors results in fewer neural crest-derived melanoblasts that nonetheless retain cell identity, localize to the follicle, and produce an altered form of melanin that likely reflects direct transcriptional repression activity of *Mash1* upon melanogenic genes, but induces hyperproliferation and transdifferentiation of non-neural crest-derived RPE precursors, thus further highlighting differences between these two classes of pigment cells.

## 711

**Desacetyl  $\alpha$ -MSH is a Partial Agonist at the Human MC1 and MC5 Receptors**

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$\alpha$ -MSH peptides are produced in the skin from the precursor protein proopiomelanocortin (POMC). While the acetylated form of  $\alpha$ -MSH is considered to be the most important agonist at the MC1-R and other MC receptors the most abundant  $\alpha$ -MSH in the skin is des- $\alpha$ -MSH. In this study we have compared the potential of these peptides as agonists at the MC1-R and MC5-R which are expressed on melanocytes and sebaceous glands, respectively. Competitive binding assays were carried out in HEK 293 cells transfected with the human MC1-R. Ac- $\alpha$ -MSH showed high affinity for the MC1-R with a  $K_i$  value of 0.135 nM. Des- $\alpha$ -MSH showed lower affinity with a  $K_i$  of 1.31 nM, respectively. ACTH1-17, which is also found in the skin and is has also been shown to act as an agonist at the MC1-R had a  $K_i$  of 0.206 nM. All three peptides activated MC1-R coupling to the cAMP pathway with Ac- $\alpha$ -MSH and ACTH1-17 being equipotent and des- $\alpha$ -MSH showing relatively weak activity. However, when present with either Ac- $\alpha$ -MSH or ACTH1-17, des- $\alpha$ -MSH reduced the stimulatory effects of these two peptides. Similar results were obtained with human melanoma cells. Unlike the  $\alpha$ -MSH peptides ACTH1-17 increased the intracellular concentration of IP3 and this effect was also reduced by des- $\alpha$ -MSH. Experiments were extended to the MC5-R transfected cells where it was shown that all three peptides increased cAMP production. Des- $\alpha$ -MSH and ACTH1-17 were equipotent but considerably less active than Ac- $\alpha$ -MSH. As seen at the MC1-R des- $\alpha$ -MSH reduced the stimulatory effect on cAMP production. However, it failed to have any effect on the action of ACTH1-17. The present results confirm that Ac- $\alpha$ -MSH is a potent agonist at the MC1-R and MC5-R. They also indicate that des- $\alpha$ -MSH, which is the most abundant  $\alpha$ -MSH peptide in the skin is a relatively weak agonist at these receptors but interestingly acts as a partial agonist to reduce the effects of Ac- $\alpha$ -MSH. If des  $\alpha$ -MSH acts in the same way *in vivo* then it could be an important regulator of melanocytes and sebaceous glands function.

## 713

**Neutralization of Melanosomal pH in P-Locus Defective Pigment Cells Enhances Melanogenesis: An Indication for P-Locus Function**

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The product of P-locus (P protein) is essential for melanogenesis and its absence or mutations lead to hypopigmentary disorders. Although P-protein has been cloned and characterised as a channel in the melanosomal membrane, its specific function has not yet been identified. The original idea that it acts as a L-tyrosine transporter has not been confirmed. More recently it has been suggested that P-protein could have a role in the determination of melanosomal pH. It has been reported that melanosomes in P-locus defective melanocytes have aberrant pH implicating an involvement of the P-protein. In this study we investigated whether neutralization of melanosomal pH would rescue melanogenesis in the P-locus defective pigment cells. We carried out experiments using the mouse melanocyte line Melan-p1 (P-locus nonfunctional) as well as the wild-type P locus cell lines Melan-a and Melan-b. Neutralisation of acidic organelles with bafilomycin A1 (BafA1) or concanamycin A (ConA) increased melanin content of Melan-p1 cells to a level comparable to that in Melan-a cells (wild type) while Melan-a cells showed no changes. Melan-b cells which have wild type P locus but severely reduced melanogenesis, as a result of mutated b locus, did not respond to neutralization. Furthermore, we observed that BafA1 and ConA restored melanogenesis in an amelanotic human melanoma line which failed to express mRNA for P-locus. We tested a panel of cDNA samples from human melanoma cell lines and found that 5 out of 5 amelanotic lines showed no expression of P-locus. We would suggest that the P-protein has a role in the neutralisation of melanosomal pH and that this change facilitates melanogenesis. P-protein is homologous to the *E. coli* Na<sup>+</sup>/H<sup>+</sup> antiporter and in the light of our findings it is reasonable to propose that the P-protein functions as a channel which acts to reduce the proton concentration inside the melanosome.

## 710

**Involvement of MITF and the PKA Signal Pathway in Regulation of Melanoblast Differentiation Induced by 8-Methoxypsoralen**

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We used melb-a cells, an immortal line of melanoblasts isolated from neonatal mouse epidermis, as a model to study the regulatory mechanisms involved in their differentiation to melanocytes following exposure to 8-methoxypsoralen (8MOP). Tyrosinase activity increased 7-fold and melanin content increased 3-fold in melb-a cells treated with 10  $\mu$ M 8MOP for 6 d compared with untreated controls. Forskolin, a PKA activator, enhanced the 8MOP stimulation of tyrosinase activity, which was higher in melb-a cells exposed concomitantly to forskolin (10  $\mu$ M) and 8MOP (100  $\mu$ M) for 6 d than in cells treated with 8MOP alone or forskolin alone ( $p < 0.01$  or  $p < 0.05$ ), respectively. A PKC activator, 1-oleoyl-2-acetyl-glycerol (OAG, at 10  $\mu$ M), did not induce differentiation of melanoblasts. Combined with results obtained by Western blot analysis, melanoblast differentiation is stimulated by 8MOP through activation of the PKA pathway rather than the PKC pathway. To elucidate what roles might be played by MITF in 8MOP induced differentiation, we used Western blot and semiquantitative RT-PCR to assess the protein and mRNA expression levels of MITF and tyrosinase in melb-a cells treated with 8MOP for 6, 24, 72 or 144 h. Incubation for 6 h with 8MOP increased MITF protein and mRNA levels, and that up-regulation was further stimulated by concomitant treatment with forskolin. After incubation with 8MOP for 72 or 144 h, levels of MITF decreased to the basal level. Tyrosinase protein expression did not increase following 8MOP treatment until 24 h, after which it was markedly increased. Maximal stimulation of tyrosinase was obtained at 6 days. These findings indicate therefore, that 8MOP initially increases MITF expression through activation of the PKA pathway. In turn, MITF binds to the tyrosinase promoter M-box, thereby stimulating tyrosinase expression and inducing melanoblast differentiation *in vitro*.

## 712

**Evidence for the Regulation of Melanogenesis by Tyrosine Hydroxylase Isozyme I and Tyrosinase in the Human Melanosome**

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Both human epidermal melanocytes and keratinocytes have the full capacity for de novo synthesis of 6(R) L-erythro 5,6,7,8, tetrahydrobiopterin, an essential reducing cofactor for the rate limiting step in catecholamine synthesis, via tyrosine hydroxylase. Earlier, catecholamine synthesis has been demonstrated in proliferating keratinocytes of the epidermis in human skin. This investigation shows, for the first time, the *in vitro* expression of tyrosine hydroxylase mRNA within the pigment forming melanocyte. Furthermore, we specifically identified tyrosine hydroxylase isoenzyme I mRNA. The localisation of the protein was demonstrated in melanosomes using immunohistochemistry and immunofluorescence double staining with the monoclonal antibody NK1/beteb directed to (pre)melanosomes as well as with antihuman tyrosine hydroxylase. These observations were further substantiated with immunogold electron microscopy yielding a melanosomal membrane association for tyrosine hydroxylase side by side with tyrosinase. Gold conjugate-labelled tyrosine hydroxylase was also detected in the endoplasmic reticulum and the *trans* Golgi network, identifying the same route of origin for this enzyme as for tyrosinase. Both tyrosine hydroxylase and tyrosinase activities were independently demonstrated in melanosomal preparations from the melanoma cell line FM94 by measurement of the catalytic release of tritiated water from 3H-L-tyrosine. The melanosomal membrane location of tyrosine hydroxylase implies a coupled tyrosinase/tyrosine hydroxylase interaction, because the latter enzyme has the kinetic potential to provide the preferred substrate and activator, L-Dopa, for tyrosinase to facilitate melanin biosynthesis. Our results support a direct function for tyrosine hydroxylase in the melanosome via a concerted action with tyrosinase to promote pigmentation.

## 714

**Neutralization of Melanosomal pH by Superoxide can be Involved in the Regulation of Pigmentation**

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UVR increases the levels of reactive oxygen species (ROS) in the skin. The first to be formed is superoxide anion ( $O_2^-$ ) and this serves as a precursor for other ROS. Although  $O_2^-$  can be cytotoxic at high concentrations, it may have physiological importance in UV mediated effects. Thus it has been reported that  $O_2^-$  stimulates melanogenesis in B16 melanoma cells. We now propose a possible mechanism as to how  $O_2^-$  acts to increase melanogenesis. The theoretical basis for our hypothesis is that as  $O_2^-$  dismutates to other ROS it scavenges protons. We have reported previously that neutralization of melanosomal pH induces an immediate increase in melanogenesis and would propose that  $O_2^-$  facilitates melanogenesis by neutralizing melanosomal pH. To test this hypothesis we examined (i) whether noncytotoxic concentrations of  $O_2^-$  will neutralize acidic organelle pH; (ii) whether  $O_2^-$  will enhance melanin production in pigment cells and (iii) whether these effects are correlated. Xanthine oxidase (XO) was used as a source of  $O_2^-$ . Non-cytotoxic concentrations of XO (20–50 mU per ml) induced a rapid and complete neutralization of acidic organelles. This was followed by increases in tyrosinase activity and melanin production. These effects were observed in mouse B16 cells, human melanoma cells and normal human melanocytes. Protein synthesis inhibition by cycloheximide had no effect on these increases. Electron microscopy revealed an accumulation of melanin and a maturation of melanosomes in response to pH neutralisation. XO did not potentiate the melanogenic effects of other substances, e.g. bafilomycin A1 which neutralised melanosomal pH supporting the idea that  $O_2^-$  acts by the same mechanism. In conclusion,  $O_2^-$  neutralizes acidic organelle pH and this change in melanosomal pH is responsible for its stimulation of melanogenesis. Thus  $O_2^-$  may have a physiological role in the UV induced tanning response. This now needs to be investigated *in vivo*.

## 715

### Melanosome Mapping by Purification of Early Stage Melanosomes

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This study reports a method for stage I/II melanosome purification and relates their characteristics and what those results imply for the regulation of melanosome biogenesis and function. Melanosomes were isolated from human MNT-1 melanoma cells following disruption of the cells with a tissue homogenizer, and separation by sucrose density gradient centrifugation. The 1.0 M sucrose fraction was found by EM to be enriched in Stage I/II melanosomes and was then further purified by free flow electrophoresis (FFE). When distribution of proteins and tyrosinase was assessed in each fraction, they were found to elute at distinct and discrete parts of the FFE gradient. The specific activity of tyrosinase was about 50 times higher than in the starting material with 40% recovery. Using EM, we found that the tyrosinase rich FFE fraction consisted of almost all Stage II melanosomes while the high protein density fraction contained mitochondria and other organelles, including Stage I melanosomes. Using melanosomal protein specific antibodies, western immunoblotting showed that the Stage II melanosome fraction was rich in tyrosinase, DCT, GP100 (as recognized by HMB45) and MART1. TYRP1 and GP100 (as detected by  $\alpha$ PEP13) was abundant in the high protein concentration fractions. Since reactivity with antibodies that recognize GP100 (i.e.  $\alpha$ PEP13 and HMB45) localized in distinct fractions, we used immuno-EM to confirm the specificity of epitope recognition by those antibodies. The immuno-reactivity of  $\alpha$ PEP13 was found on the exterior surface of the melanosomal membrane, while in contrast, reactivity with HMB45 was seen only with the internal matrix of melanosomes following proteolytic cleavage of GP100. These results suggest that Stage I melanosomes initially contain only TYRP1 and GP100, and that tyrosinase, DCT and MART1 are detectable only after the formation of Stage II melanosomes which may depend on the proteolytic processing of GP100.

## 717

### Keratinocytes Play a Role in Regulating Distribution Patterns of Recipient Melanosomes

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Melanosomes in keratinocytes of Black skin are larger and distributed individually, whereas those within keratinocytes of Caucasian skin are smaller and distributed in clusters. This disparity contributes to differences in skin pigmentation and photoprotection, but the control of these innate melanosome distribution patterns is poorly understood. To investigate this process, cocultures were developed so that melanosome transfer from the melanocyte to the keratinocyte occurred. This transfer process was confirmed by confocal microscopy using melanocytes labelled with a fluorescent and electron microscopy (EM). Co-cultures were established using melanocytes and keratinocytes derived from different pigimentary backgrounds and examined by EM. Melanosomes transferred to keratinocytes were categorized as individual or in various clusters. Melanosome size was also determined for individual and clustered melanosomes. Results indicate that in our model system, melanosomes in keratinocytes from different pigimentary backgrounds have a combination of clustered and individual melanosomes. However, when keratinocytes from dark skin were cocultured with melanocytes from (a) dark or (b) light skin, recipient melanosomes were individual vs. clustered at (a) 77.6% vs. 22.4% and (b) 63.5% vs. 36.5%, respectively. In contrast, when keratinocytes from light skin were cocultured with melanocytes from (c) dark or (d) light skin, recipient melanosomes were individual vs. clustered at (c) 33.5% vs. 66.5% and (d) 38.7% and 61.3%, respectively. These results indicate that recipient melanosomes, regardless of origin, are predominantly distributed individually by keratinocytes from dark skin; and those from light skin in membrane-bounded clusters. In contrast, melanosome size did not differ between distribution patterns. These results suggest that regulatory factor(s) within the keratinocyte determine recipient melanosome distribution patterns.

## 719

### Synchronized Maturation of Tyrosinase by Temperature Shift

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Tyrosinase is localized in lysosomes when expressed in nonmelanosomal cells. To elucidate the intracellular transport mechanism of tyrosinase, we have developed a novel experimental system using a temperature-shift technique. When wild type tyrosinase was expressed in SiHa cells at 40°C, tyrosinase accumulation was observed in the reticular network but not in granular structure of lysosome by indirect immunofluorescence. To examine if the transport arrest in the endoplasmic reticulum (ER) is released by lowering the temperature, we added cycloheximide to inhibit protein synthesis and analyzed the transport during incubation at 37°C. Upon incubation at 37°C, exit from the ER was clearly detected, and tyrosinase was concentrated at the juxtanuclear region at 30 min after the temperature shift. Additional 2 h incubation at 37°C allowed majority of the expressed molecules to reach granular lysosomal structures. We next determined the enzyme activity of the cell extracts to see if the transport arrest at 40°C was caused by misfolding. Indeed, little DOPA oxidase activity was detected when incubated at 40°C. However, the oxidase activity became detectable by incubation of the cells at 37°C, suggesting that the expressed tyrosinase was reversely arrested in the ER due to aberrant folding at 40°C by the quality control machinery. Thus, we conclude that maturation of tyrosinase could be synchronized by temperature-shift, providing a novel approach to analyze intracellular transport mechanism of melanosome-associated proteins.

## 716

### Induction of Protein Kinase C- $\beta$ and Tyrosinase mRNA by cAMP-Dependent Pathway in Cultured Human Melanocytes

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Protein Kinase C- $\beta$  (PKC- $\beta$ ) and cAMP are known to modulate pigmentation. In cultured human melanocytes, cAMP has been shown to induce the level of PKC- $\beta$  protein, increasing tyrosinase activity. To determine if the cAMP-induced increase in PKC- $\beta$  protein is due to increased transcription, paired cultures of human melanocytes were examined after removing bovine pituitary extract and dibutyryl cAMP, agents routinely added to the medium to support the growth of melanocytes but which raise cAMP above physiologic levels. Cultures were then treated with 100  $\mu$ M isobutyl methyl xanthine (IBMX) or vehicle for 72 h, by which time IBMX is known to increase PKC- $\beta$  protein. Cells were harvested and PKC- $\beta$  mRNA was measured using Northern blot analysis. The level of PKC- $\beta$  mRNA in IBMX-treated cells was 3 times higher in vehicle-treated cells. The half-life of PKC- $\beta$  mRNA in IBMX-treated cells, determined in duplicate cultures of melanocytes using the transcription inhibitor 5,6-dichloro-1- $\beta$ -D ribofuranosylbenzimidazole (DRB) at 25 mg per ml for 0, 4, 8, and 22 h, was greater than 24 h. In parallel the level and half-life of tyrosinase mRNA was determined. Tyrosinase mRNA in IBMX-treated cells was 2-3 times higher than in vehicle-treated cells; in vehicle treated cells, the half-life of tyrosinase mRNA was 6-8 h, as previously reported in murine melanoma cells but, in IBMX treated cells, the half-life was greater than 22 h, suggesting that cAMP increases tyrosinase mRNA stability. Thus, the cAMP-dependent pathway increases human pigmentation by up regulating PKC- $\beta$  and tyrosinase mRNAs.

## 718

### The Role of Extension Gene in Bovine Coat Color Determination. Non-Black Phenotypes are Associated with Transcripts Spliced in the 3'UT Region

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We established the complete structure of the bovine extension gene encoding the G-protein coupled melanocortin-1 receptor, a seven transmembrane domain protein that controls the signaling pathway of melanin synthesis in melanocytes. As in human homologue gene, the active promoter region seems to be associated to the presence of E-boxes and Sp1 binding sites. The 5'-upstream region of the four alleles previously identified (E, ED, E1 and e) doesn't present any polymorphism that can be related to the various eumelanin and/or pheomelanin coat color phenotypes. Interestingly, two types of transcripts are detected in skin samples. A first primary transcript, which has a nucleotide sequence identical to the corresponding genomic DNA, is observed among all individuals whatever their coat-color phenotypes. In addition, a spliced transcript is observed only among the skin of the nonblack individuals and in the noncolored skin regions of spotted animals. Spliced transcripts have lost an alternative intronic sequence of about 200 bp (193-218) overlapping the typical 3' downstream position of RNA regulatory elements that control mRNA stability. The size of introns and exon-intron junctions are specific of each coat-color selected bovine population. The significance of this post-transcriptional process is discussed considering the role of RNA binding proteins in translation and turnover of messenger RNA.

## 720

### Proper Folding and ER to Golgi Transport of Tyrosinase are Induced by its Substrates, DOPA and Tyrosine

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Tyrosinase is essential for pigmentation, and is a source of tumor-derived antigenic peptides and cellular immune response. Wild type tyrosinase in melanoma cells and loss of function albino mutants in untransformed melanocytes are misfolded proteins that are retained in the endoplasmic reticulum (ER), subsequently retranslocation to the cytosol and targeted to proteolytic degradation by the 26S proteasome. Here we demonstrate that tyrosinase catalytic activation is required for proper folding and ER exit. The cofactor and substrate DOPA, in the presence of tyrosine, induced in melanoma cells within hours of exposure a transition in tyrosinase from the misfolded to the native form, that is resistant to proteolysis, competent to exit the ER, and able to produce melanin. Since tyrosinase's enzymatic activity is induced by DOPA, we propose that proper folding of the wild type protein, just like mutant forms, is tightly linked to its catalytic state. Loss of pigmentation therefore in tyrosinase-positive melanoma cells is a consequence of tumor induced metabolic changes that suppress tyrosinase activity within these cells. Since solid human tumors, including melanomas, induce acidification of their extracellular milieu, largely in response to anaerobic glycolysis and accumulation of lactic acid, we suggest that these metabolic changes affect tyrosinase activity within the ER.

## 721

**Abnormal Tyrosinase Processing and Fate in Melanocytes Lacking the Pink-Eyed Dilution Gene**

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Pink-eyed dilution gene (*p*) mutations result in Oculocutaneous Albinism Type 2, the most common form of albinism. The processing of tyrosinase, which catalyzes the rate limiting reaction in melanin synthesis, was investigated in melan-p1 melanocytes which are null at the *p* locus. Electron Microscopy detected a high numbers of 50 nm vesicles which contain active tyrosinase in Melan-p1 cells, but only a small number are present in wildtype melanocytes. Triton-X114 phase separation and Endoglycosidase H digestion show that tyrosinase processing is altered in the absence of *p* expression. A fraction of tyrosinase is retained in the Endoplasmic Reticulum while the remainder undergoes further processing and appears to be proteolyzed at an increased rate. The proteolyzed tyrosinase is no longer membrane bound, but remains enzymatically active and much of it is secreted into the culture medium of melan-p1 cells. Small gradient fractions enriched for small vesicles contained predominantly proteolyzed tyrosinase. Our results suggest that an increased proportion of tyrosinase is misfolded and prematurely proteolyzed in melan-p1 melanocytes. Much of the proteolyzed tyrosinase is transported in small vesicles and is subsequently secreted rather than reaching the melanosome. The pink-eyed dilution protein may thus play a role in the ER processing of tyrosinase.

## 723

**Tyrosinase and Tyrosinase Related Protein-1 (TRP-1) Require rab7 for the Intracellular Transport from TGN to Melanosomes, Passing Through Early and Late Endosomes**

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Tyrosinase and tyrosinase-related proteins (TRPs) are glycosylated membrane-bound melanogenic proteins. They are transported from endoplasmic reticulum (ER) to *trans* Golgi network (TGN) and finally to melanosomes. Previously, we have proposed that late endosomes and rab7 are also involved for tyrosinase transport between TGN and melanosomes. In order to further investigate the vesicular transport process of tyrosinase and TRP-1, we expressed these melanogenic proteins, by using recombinant adenoviruses, in amelanotic SK-MEL-24 human melanoma cells in the presence or absence of dominant-negative rab7 mutant (rab7N125I). Tyrosinase and TRP-1 became detectable in 4–5 h within the cytoplasm and, in the case of Ad-HT (tyrosinase) infection, melanin production was observed from 24 h. To further clarify the intracellular localization and the involvement of early late endosomes for the transport of tyrosinase and TRP-1 to melanosomes, SK-MEL-24 cells were infected with Ad-lacZ (fA-galactosidase), Ad-rab7 wt (wild type rab7) or Ad-rab7N125I for 48 h and then with Ad-HT (tyrosinase) or Ad-TRP-1 (TRP-1). Specific intracellular localization was identified by mono- or poly clonal antibody specific for detecting tyrosinase or TRP-1, respectively. They were also processed to double immuno-staining using early and late endosome markers. We found that (a) melanin synthesis was much lower in Ad-rab7N125I mutant than in Ad-rab7 wt infectants (b) the transport of tyrosinase and TRP-1 from TGN to late endosomes was blocked in the rab7N125I mutant infectants, and (c) they were localized in the enlarged granules which also expressed EEA-1, early endosome marker. Our results strongly indicate that transport of tyrosinase and TRP-1 requires rab7, passing through compartments of both early and late endosomes before targeted to melanosomes.

## 725

**Melanocyte Dysfunction in Hermansky-Pudlak Syndromes (HPS-1 & 2)**

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Hermansky-Pudlak Syndrome Type 1 (HPS-1) results from mutations in the HPS gene that encodes a 79.3-kDa soluble protein that can form part of a high molecular weight complex of unknown function. Melanocytes cultured from HPS-1 demonstrate suppressed tyrosinase activity and altered localization of melanocyte specific proteins. Tyrosinase, tyrosinase related protein-1 (TRP-1), and ME-491 were localized to large, membranous complexes throughout the melanocytes and relatively absent from melanosomes. Recapitulation of the hypomelanosis, formation of large membranous complexes, and altered trafficking of tyrosinase in melanoma cells transfected with antisense HPS-1 cDNA confirmed that the HPS-1 protein is required for successful targeting of melanocyte specific proteins to the premelanosome. Localization studies indicate that HPS-1 may be associated with a cisternal network outside of the Golgi zone in normal melanocytes. HPS-2 results from mutations in the  $\beta$ -3 A subunit of the heterotetrameric adaptin complex AP-3. Melanocytes from HPS-2 demonstrated a dramatically reduced expression of  $\beta$ -3 A. HPS-2 melanocytes exhibited normal distribution of TRP-1, LAMP-1, LAMP-3 and rab27 throughout the cell body and along the dendrites. In contrast, tyrosinase expression was reduced and predominantly restricted to the perinuclear regions of HPS-2 melanocytes. Tyrosinase juxtapositioned to and localized in large vesicles resembling late endosomes as demonstrated by DOPA histochemistry at the EM level. This abnormal localization of tyrosinase could be corrected with transfection of the AP-3 cDNA into HPS-2 melanocytes. These data suggest that tyrosinase trafficking to the melanosome is regulated in part by AP-3, independent of TRP-1.

## 722

**Interaction of Tyrosinase Related Protein-1/gp75 with PDZ-domain Protein GIPC: Relationship to Biosynthesis and Intracellular Sorting**

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Interactions of cytosolic proteins with determinants on the cytoplasmic domains of vesicular membrane proteins play a critical role in intracellular sorting, vesicular targeting and biogenesis of organelles. Cytoplasmic domains of melanosomal membrane proteins tyrosinase and tyrosinase related proteins (TRPs) contain signals for melanosome targeting. We employed the yeast two-hybrid screen and identified a protein that interacts with the cytoplasmic domain of gp75/TRP-1, the most abundant melanosomal membrane protein. The gp75 interacting protein (GIP) encoded by a partial 1.4 kb cDNA fragment has a single PDZ domain. Immunoprecipitation of gp75 from detergent extracts of COS cells transfected with epitope-tagged GIP and gp75 efficiently coprecipitated GIP. Deletion of carboxyl (C) terminal amino acids QSVV of gp75 abolished its interaction with GIP. Substitution of amino acids residues in the lysosomal/melanosomal sorting signal of gp75 did not affect this interaction. GIP did not bind tyrosinase tail which lacks C-terminal QSVV sequence, but tyrosinase tail with two C-terminal residues mutated to valines bound to GIP efficiently. Immunofluorescence analysis of COS cells transfectants showed colocalization of gp75 and GIP in a subset of intracellular vesicles. Cotransfection with mutant gp75 proteins showed that binding to gp75 determines cellular distribution of GIP. Amino acid sequence of GIP is identical to the sequence of GIPC, a protein that binds to RGS-GAIP (a regulator of G protein signaling) localized to clathrin-coated vesicles and to the sequence of SEMCAP-1, a neural semaphorin binding protein. Thus, specific interaction of a multifunctional PDZ domain protein with gp75 suggests that gp75 and presumably other TRPs play novel nonenzymatic roles in melanogenesis through interaction of cytosolic proteins with their tails.

## 724

**Melanosomes from Human Melanocytes Express the GTP-Binding Protein cdc42 and cdc42-Effector Molecules PAK-1 and N-WASP: Analysis of the Role of cdc42 in Melanosome Transfer to Keratinocytes**

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Melanosomes are specialized melanin-containing organelles that arise from the Golgi apparatus, are transported on microtubules to the melanocyte (MC) dendrite tip and then are transferred to keratinocytes (KC). The mechanisms of melanosome transfer to KC are poorly understood. We present evidence to suggest that melanosome transfer to KC is mediated by the GTP-binding protein cdc42, a protein involved in actin-based filopodia formation in neuronal cells, and by the cdc42 effector proteins PAK-1 and N-WASP. Human MC-KC cocultures were placed in a heated chamber perfused with media and viewed under Nimsarky optics on a Nikon Eclipse 800 microscope at 100X. Sequential digital images were obtained with a Spot camera at 8-s intervals. Images obtained with 10 pixels/micron were further processed using Matlab and converted to Quick Time Movies. Melanosomes were observed in filopodia-like structures arising from the tips of the dendrites. Filopodia were as long as 16 microns in length, and were dynamically active. Bi-directional movement of melanosomes along filopodia and insertion of filopodia into KC was observed with subsequent transfer of melanosomes to KC. Confocal microscopy of MC dual labeled for actin and melanosomal proteins (myosin V and/or tyrosinase related protein-2) confirmed the presence of melanosomes in filopodia as did electron micrographs of MC-KC cocultures. Because of the well-documented role of cdc42 and its effector proteins N-WASP and PAK-1 in filopodia formation in neuronal cells, we examined the expression of these proteins in human MC by immunofluorescence staining/Confocal microscopy and Western blotting. Cdc42 colocalized with tubulin and  $\beta$ -COP (a Golgi maker), as well as melanosomes. Melanosomes isolated from human MC by sucrose density ultracentrifugation expressed the cdc42 effector proteins PAK1 and N-WASP by Western blotting, as well as cdc42. We suggest that melanosomes act as chaperones to escort N-WASP and PAK1 to the dendrite tip, which, in combination with cdc42, induce filopodia formation and transfer of melanosomes to KC. In combination with our previous work showing SNARE proteins and rab3a on melanosomes, proteins involved in membrane fusion and exocytosis, respectively, our observations suggest a novel model for melanosome transfer to KC.

## 726

**Intracellular Distribution and Late Endosomal Effects of the Ocular Albinism 1 Gene Product: Consequences of Disease-Causing Mutations and Implications for Melanosome Biogenesis**

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In order to elucidate the molecular pathogenesis of ocular albinism type 1, we examined the intracellular distribution and subcellular effects of wild type Oa1 and Oa1 constructs reflecting mutations causing X-linked ocular albinism. To this end, we employed constructs containing either wild typemurine Oa1, or Oa1 constructs containing missense mutations, fused to green fluorescent protein in a COS cell expression system. Comparison with different organelle markers revealed that wild type Oa1-GFP localized to the late endosomal/lysosomal compartments. Some Oa1 constructs bearing disease-causing mutations failed to exit the ER (Class I mutants), while other mutants partially (Class II mutants) or fully (Class III mutants) exited the ER and trafficked to endolysosomal compartments. We observed that expression of wild type Oa1-GFP in COS cells caused an enlargement of late endosomes, and redistribution of the late endosomal markers mannose-6-phosphate receptor (M6PR) and lysobisphosphatidic acid (LBPA). Only a subset of the mutants (Class III) examined were capable of causing LE enlargement, and none of the mutants displayed the full range of effects on M6PR and LBPA exhibited by wild type Oa1. The effects of Oa1 on LE structure and content are thus likely to reflect an important biological property of Oa1. In its absence, late-endosome derived melanin macroglobules may accumulate at the expense of normal melanosomes, thus leading to albinism. Our results suggest that the late endosomal compartment plays a central role in melanosome biogenesis and that Oa1 controls a critical step in the process.

## 727

### Identification of a Novel Moab J1 Which Recognizes Luminal Structure of Lysosomes Common To Melanosomal Compartment in Late Stage of Melanogenesis

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Melanogenesis cascade may directly/or indirectly link to the dynamics of endosome-lysosome biogenesis. Our recent gene-transfection studies suggested that tyrosinase and tyrosinase-related proteins (TRPs) are transported from *trans* Golgi network (TGN) to late endosomes and subsequently to melanosomes. However, little is known about how and to what extent the biogenesis of melanosomes and lysosomes are related. The objective of this study is to further identify the involvement of endosome-lysosome system in melanosome biogenesis. Specifically this study utilized a new antibody "J1", which we identified in the process of developing monoclonal antibodies (MoAbs) against human melanosomes. MoAb J1 appeared to identify a new luminal component of lysosomal granules. It was also reactive with not only melanotic and amelanotic melanoma cells (e.g. MeWo, SK-MEL-23 and SK-MEL-24) but also nonmelanoma cells (e.g. MRC5 fibroblasts and HeLa cells). To identify the J1-positive structures, we sequentially stained the cells with a series of well-characterized probes including Alexa-488 conjugated pepstatin for lysosomal luminal content and antibodies against Ig85 (lysosomal membranes), syntaxin 8 (late endosomes), EEA1 (early endosomes), KDE1 (ER) as well as tyrosinase (melanosomes). We found that (1) J1 antigen was expressed in granular structures which colocalized with lysosomal luminal protein in the entire cytoplasm of melanoma and nonmelanoma cells, but more extensively melanotic melanoma cells (2) J1 also stained most of the tyrosinase-positive granules (3) J1 antigen was coexpressed with many (c. 30–40%) of Ig85 positive granules, and (4) J1 did not stain structures which were positive with late/early endosomal or ER markers. Based upon these results, we conclude that J1 possesses a novel antigen epitope that is expressed in the melanogenic cascade common to a luminal component of both melanosomal and lysosomal compartments, being specifically involved in the late stage of melanosome biogenesis.

## 729

### DCoH/PCD (Dimerization Cofactor of Hepatocyte Nuclear Factor 1/pterin-4a-carbinolamine Dehydratase) is Crucial for Pigmentation in Xenopus and Overexpressed in Primary Human Malignant Melanoma

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DCoH/PCD is a positive cofactor of the HNF1 homeobox transcription factors as well as an enzyme catalyzing the regeneration of tetrahydrobiopterin. Dysfunction of DCoH/PCD is associated with the human disorders hyperphenylalaninemia and vitiligo. In this study loss of function experiments using DCoH/PCD specific antibodies injected into fertilized *Xenopus* eggs demonstrated that the protein is absolutely necessary and also sufficient for pigment formation. In normal human skin DCoH/PCD protein was weakly expressed in the basal layer of the epidermis, whereas high protein levels were detectable in several melanoma cell lines and primary malignant melanoma. The DCoH/PCD overexpression was restricted to and identified all of the melanoma cells. Comparison with the commonly used melanoma markers S100 and HMB45 demonstrates that DCoH/PCD had an overlapping but distinct expression pattern. In addition to human colon cancer, this is the second report about the overexpression of DCoH/PCD in human tumor cells indicating that the protein may play a role in carcinogenesis.

## 731

### Neural Factors Play a Role in the Pathogenesis of Vitiligo

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In order to confirm a relationship between vitiligo and the activity of mono-aminergic systems, we measured by high-pressure liquid chromatography and electrochemical detector (HPLC-ED): (1) plasma levels of some catecholamines such as norepinephrine (NE), epinephrine (E) and dopamine (DA), their precursor 3,4 dihydroxyphenylalanine (DOPA), their metabolites [normetanephrine (NMN), metanephrine (MN), 3-methoxy-4-hydroxyphenyl-glycol (MHPG), vanillylmandelic acid (VMA), homo-vanillic acid (HVA)] and 5-hydroxyindolacetic acid (5-HIAA), as the major metabolite of serotonin; and (2) urinary excretion values of the same parameters plus vanillylmandelic acid (VMA) in 25 healthy subjects and in 47 patients suffering from nonsegmental vitiligo at different stages of disease. Plasma and urinary mean values of the biological markers were higher in vitiliginous subjects, mainly in the subgroup of patients at an active phase of disease (n=37/47), than in controls. The difference was significant for all parameters, except for plasma and urinary HVA. The patients with progressive vitiligo and at its more recent onset (< 1 years) showed increased levels of all considered parameters than longer-term sufferers. No significant differences were observed in patients subdivided according to the type of vitiligo or the age of onset. Significant intercorrelations were noted both in plasma and in urine among noradrenergic markers as well as between HVA and 5-HIAA. The higher catecholamine and metabolite levels indicate that the activity of monoaminergic systems is increased in the early phase of vitiligo, a fact which may probably have a role in the onset of the disease itself.

## 728

### Expression of Microtubule-Associated Protein 2 in Benign and Malignant Melanocytes: Implications for Differentiation and Progression of Cutaneous Melanoma

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Cutaneous melanocytic neoplasms are known to acquire variable characteristics of neural crest differentiation. Melanocytic nevus cells in the dermis and desmoplastic melanomas often display characteristics of nerve sheath differentiation. The extent and nature of neuronal differentiation characteristics displayed by primary and metastatic melanoma cells is not well understood. Here, we describe induction of a juvenile isoform of microtubule-associated protein 2 (MAP-2c) in cultured metastatic melanoma cells by the differentiation-inducer hexamethylene bisacetamide (HBMA). Up-regulation of this MAP-2 isoform, a marker for immature neurons, is accompanied by extended dendritic morphology and down-regulation of the melanocyte-differentiation marker tyrosinase-related protein 1 (TYRP1/gp75). In a panel of cell lines that represent melanoma tumor progression, MAP-2c mRNA and the corresponding ~70 kDa protein could be detected predominantly in primary melanomas. Immunohistochemical analysis of 61 benign and malignant melanocytic lesions showed abundant expression of MAP-2 protein in melanocytic nevi and in the *in situ* and invasive components of primary melanoma, but only focal heterogeneous expression in a few metastatic melanomas. In contrast, dermal nevus cells and the invasive cells of primary melanomas, which stained positive for MAP-2, were TYRP1 negative. This reciprocal staining pattern *in vivo* is similar to the *in vitro* observation that induction of the neuronal marker MAP-2 in metastatic melanoma cells is accompanied by selective extinction of the melanocytic marker TYRP1. Our data show that neoplastic melanocytes, particularly at early stages, retain the plasticity to express the neuron-specific marker MAP-2. These observations are consistent with the notion that both benign and malignant melanocytes in the dermis can express markers of neuronal differentiation.

## 730

### An Alternative Approach to Depigmentation by Soybean Extracts via Inhibition of the PAR-2 Pathway

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The Protease-activated receptor-2 (PAR-2) is expressed on keratinocytes but not on melanocytes. PAR-2 is involved in the regulation of pigmentation, affecting melanosome phagocytosis by keratinocytes. Inhibition of PAR-2 activation by serine protease inhibitors results in skin lightening *in vivo*, suggesting a new class of depigmenting mechanism and agents. Natural agents that affect the PAR-2 pathway were tested for their possible modulation of pigmentation. Here we show that soymilk and the soybean-derived serine protease inhibitors STI and BBI inhibit PAR-2 cleavage, affect cytoskeletal and cell surface organization and reduce keratinocyte phagocytosis. The depigmenting activity of these agents and their ability to prevent UV induced pigmentation are demonstrated both *in vitro* and *in vivo*. These results suggest that the inhibition of the PAR-2 pathway by soymilk may be used as a natural alternative to skin lightening.

## 732

### Recombined Human/Porcine Skin Methodology for Vitiligo

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Vitiligo is characterised by the loss of skin pigmentation due to the destruction of melanocytes. The treatment is usually difficult. For stable cases the melanocyte transplantation is the method of choice. The newly developed treatment with recombined human/porcine skin methodology permitting easy handling of the graft is described in the present work. In vitiligo patients, autologous epidermal cells were obtained from pigmented thin skin biopsies. The cells were cultured on a dried cell-free porcine dermis with the 3T3 feeder layer technique. After 10 days melanocytes were regularly dispersed in confluent keratinocyte cultures. Upside-down delivery of epidermal cells was used. The epidermal layer was directly applied onto the dermabrased vitiligo lesion with porcine dermis covering the lesion. The pigmentation started to be visible 4–6 weeks after grafting. After using of the above described methodology the pigmentation appeared in the range of 65–80% of the grafted area. Additional UVA irradiation enhanced the treatment success up to 100%. The surgical vitiligo treatment appears to be a reasonable method of choice in stable vitiligo cases of a disease duration for at least two years which means for approximately 5% of all vitiligo patients.

## 733

**Treatment of Vitiligo with Broadband Ultraviolet B**

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While oral PUVA remains the most popular therapeutic modality for vitiligo, recent reports have shown that narrowband UVB can also induce significant repigmentation. In this study, we attempted to evaluate the efficacy of broadband UVB on actively spreading vitiligo. Nine patients with actively spreading vitiligo were exposed to broadband UVB two to three times per week at a starting dose of 20–30 mJ per cm<sup>2</sup>. Radiation was increased 10 mJ per cm<sup>2</sup> per session with adjustments for symptomatic erythema or missed visits. The response to treatment and side-effects were assessed at each visit. Broadband UVB halted the progression of vitiligo in all nine patients and induced early repigmentation after 6–12 treatments (2–4 weeks). After 2–8 months of treatment, eight out of nine patients achieved good (51–75%) or excellent (76–100%) response. The percent of repigmentation varied with length of treatment and anatomic site. This study confirms the only published report that broadband UVB is effective on actively spreading vitiligo. Since it is more cost effective than narrowband UVB and has numerous advantages to oral PUVA, broadband UVB may offer an alternative for future treatment of vitiligo.

## 735

**Nevogenesis and Differential Expression of Matrix Metalloproteinases**

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Congenital nevi are constituted by pigment cells bearing common features with melanocytes but showing altered differentiation leading to nesting and dermal location. In a reconstructed epidermis seeded with a combination of keratinocytes and various sources of pigment cells, we have studied the formation of nests and the dermal passage of those cells in correlation with the secretion of matrix metalloproteinases (MMP2 et 9). Dermal nevus cells were shortly cultured, with normal melanocytes and melanoma cells used as controls. In reconstructs, a positive correlation was established between the increasing percentage of seeded nevocytes and the pigmentation of reconstructs as well as the clustering of cells in junctional nests. However, the presence of nevocytes in the dermis of reconstructs was never detected during the time-span of the experiments. MMP 9 was never expressed in congenital nevus cells whereas MMP2 was constitutively expressed by all strains of congenital nevus cells. Previous studies had shown that melanocytes produced comparable amounts of both MMP2 and MMP9, and that Bowes melanoma cells secrete a marginal level of MMP2. So, nevus cells would correspond to an intermediate status of differentiation between normal melanocytes and melanoma cells. Activation of MMPs by a cofactor or activation of another pathway seems necessary to provoke the dermal passage of nevus cells.

## 737

**Computerized Digital Image Analysis can Improve the Detection of Potentially Dangerous Pigmented Skin Lesions**

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Melanoma is completely curable if resected early. Unfortunately, early melanoma can lack the clinical features that are used for diagnosis. Diagnostic accuracy can be improved by dermoscopy, but this requires experience which is difficult to master. Computerized digital analysis instruments have now been developed to assist in determining whether or not a pigmented lesion is potentially dangerous and needs to be biopsied. One such instrument is the DermoGenius System (Rodenstock Precision Optics, Munich, Germany). Lesions are scanned and the digital images analyzed using an algorithm that assesses asymmetry of color and shape, border qualities, color variegation and homogeneity, and structural components. A score = 2 suggests an increased chance of melanoma and warrants a biopsy. To evaluate whether this instrument can improve the management of pigmented lesions, we biopsied 18 pigmented lesions that appeared clinically benign to an experienced dermatologist. The lesions were biopsied based on having a computer-generated score of = 2.0. The lesions were biopsied based on a computer-generated score suggestive of an increased chance of the lesion being melanoma. The software used to generate the score was a precursor (beta) version of the final one to be developed. Histologically, 1 lesion was a dysplastic nevus with cytotoxic atypia that should have been removed. Nine of the lesions were ordinary dysplastic nevi, and the balance were benign intradermal nevi. These results suggest that computerized image analysis can improve the evaluation of pigmented skin lesions by recommending the biopsy of clinically unuspicious but potentially dangerous lesions that might have otherwise been neglected.

## 734

**Photoprotective Melanogenesis Induced by Bicyclic Monoterpene Diols**

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The purpose of this study was to investigate agents that induce a natural tan without exposure to damaging solar radiation. Previous studies have shown that a variety of aliphatic and alicyclic diols induce melanogenesis in S91 mouse melanoma cells, normal human epidermal melanocytes, and guinea pig skin (*J Invest Dermatol* 10:428–437, 1998). The most potent compounds examined previously were the bicyclic monoterpene (BMT) diols 5-norbornene-2,2-dimethanol and 2,3-*cis*/*exo*-pinanediol. These compounds induced melanogenesis in S91 cells via the nitric oxide/cyclic guanosine monophosphate/protein kinase G pathway (*Pigment Cell Res* 12:36–47, 1999). More recently, novel BMT diols have been synthesized that are 5- to 10-fold more potent than previous compounds with regards to induction of melanogenesis and nitric oxide. These new compounds include 2,2-dimethyl-3-(2,3-dihydroxy-propan-3-yl)-norbornane (1S,5S)-(–)-6,6-dimethylbicyclo[3,1,1]heptane-2,2-dimethanol, and (1R)-(–)-6,6-dimethylbicyclo [3,3,1]hept-2-ene-2,3-dimethanol. Pilot studies using 2,3-*cis*/*exo*-pinanediol and 2,2-dimethyl-3-(2,3-dihydroxy-propan-3-yl)-norbornane showed that these induce tanning when encapsulated in liposomes and applied at 20 mM to human skin. Induction of melanogenesis by 250 and 500  $\mu$ M 2,3-*cis*/*exo*-pinanediol in S91 cells was associated with 20% reductions of cyclopurimidine dimers produced by subsequent irradiation with 125 J per m<sup>2</sup> UVB. This suggests that tanning induced by BMT diols may be photoprotective in human skin.

## 736

**Mutagenesis in Dysplastic (Atypical) Nevus Cells may be Caused by Increased Oxidative Stress**

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There are indications that in dysplastic (atypical) nevi the composition of melanin is abnormal. We utilized X-ray microanalysis to measure different elements in the melanosomes of histologic preparations of 12 atypical nevi and compared the results with those obtained from the melanosomes of normal skin of the same individuals. The atypical nevi originated from heterogeneous group of patients with solitary or multiple nevi, with or without the history of familial melanoma. Our results show that the melanosomes of clinical atypical nevi synthesize significantly more pheomelanin. Since polymer melanin contains many carboxyl groups it can function as a weak cation exchanger. In many cases of dysplastic nevus cells we found highly elevated melanosomal concentration of calcium and iron. This is the indication of increased concentration of these metal ions in the cytoplasm. The elevated concentrations of calcium and iron can be explained by the existence of chronic oxidative stress in these cells. Using a model situation we demonstrate that the increased iron concentration can be caused by iron liberation from ferritin molecules by leaking pho- and/or eumelanin precursors. Liberated iron ions can bind to phosphate groups of DNA. Such situation is connected with the increased risk of oxidative DNA damage and mutagenesis.

## 738

**Real Time Microscopical Observations of Melanocytes and their Responses to  $\alpha$ -MSH in a Transparent Skin Equivalent**

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It is known that  $\alpha$ -MSH stimulates skin pigmentation in humans. While this involves an increase in melanogenesis in melanocytes, new evidence suggests that the pigmentary response to  $\alpha$ -MSH also involves effects on melanocyte dendricity, motility and interactions with keratinocytes. These processes have been studied in monolayer cultures but not in 3D models which resemble more closely *in vivo* conditions. The aim of this study was to investigate whether transparent skin equivalents (MelanoDerm(tm)), containing melanocytes, can be used to monitor these parameters and any effects that  $\alpha$ -MSH may have. Initially we carried out immunohistochemical characterisation of the skin equivalent using mAbs Mel-2, Mel-5, NKI-beteb, NCL-c-kit and anti-E-Cadherin. The patterns of staining for all five markers was similar to those seen in human epidermis except for reduced expression of c-kit in the skin equivalent. We went on to measure tyrosinase activity. Labelled L-tyrosine was added to the skin equivalents and tritiated water production measured in the presence and absence of 10 nM  $\alpha$ -MSH on days 1, 2 and 3. These experiments showed that skin equivalents, prepared with melanocytes from Asian donors, were melanogenically active and responsive to  $\alpha$ -MSH. Real time observations of melanocytes in the skin equivalent were carried out at 400 $\times$  magnification using time lapse photography (8 frames per hour) over 24 h. Melanocyte motility, dendricity and motility of dendrites was observed. Preliminary results indicated that  $\alpha$ -MSH reduces the motility of the melanocyte cell body but increases the numbers and pulsatile behaviour of the dendrites. Our results show that melanocytes in MelanoDerm skin equivalents express melanocyte markers, are melanogenically active, capable of responding to MSH and suitable for time lapse observations. In conclusion, the transparent skin equivalent may prove to be a useful experimental model for studying pigmentary responses.



## 739

**Restoration of Pigmentation in Cutaneous Wounds by Titration of Melanocytes into Cultured Skin Substitutes Grafted to Athymic Mice**

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Hypopigmentation after grafting of cultured skin substitutes (CSS) results in long-term morbidity to patients. Hypothetically, morbidity from hypopigmentation may be reduced by addition of sufficient melanocytes to restore uniform skin color. To address this hypothesis, CSS were prepared from cultured human keratinocytes and fibroblasts attached to collagen-based substrates. Cultured human melanocytes from a black donor (melanin content = 90.8 µg per 10<sup>6</sup> cells) were added to keratinocytes (1.1 × 10<sup>6</sup> cells per cm<sup>2</sup>) of CSS at inoculation densities of 0.0%, 0.01%, 0.10%, and 1.0%, incubated 18 days and grafted to full-thickness skin wounds in athymic mice. On culture day 14 *in vitro*, and 12 weeks after grafting, pigmentation was assessed with a Minolta Chromameter to determine surface darkness. Pigmented areas of healed CSS were determined by tracing and planimetry at 12 weeks after grafting. Data from each condition were compared using one-way ANOVA to identify significant differences (*p* < 0.05 vs. all other groups). Data are expressed as Chromameter L\* values (black = 0; white = 100), and percentage pigmented area. Chromameter L\* values were significantly lower for the 1% group compared to other groups at 14 days *in vitro*, and significantly greater for either the 0.0% or 0.01% group compared to other groups at 12 weeks after grafting. Similarly, % pigmented area was significantly lower than all other groups for CSS with 0.0% or 0.01% melanocytes, was 100% pigmented in CSS with 1% added melanocytes, and was proportionate to melanocyte density. These results demonstrate that cutaneous pigmentation can be restored with a melanocyte density of 1% of keratinocytes (1.1 × 10<sup>4</sup> cells per cm<sup>2</sup>) in CSS, and that clinical morbidity from hypopigmentation can be reduced or eliminated by addition of cultured melanocytes to CSS used for wound repair.

## 741

**Calgranulin C is Overexpressed in Psoriatic Skin *In Vivo***

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The calgranulins are members of the S-100 family of calcium-binding proteins potentially involved in the pathogenesis of psoriasis. Since the calgranulins A and B have been found up-regulated on the mRNA and protein level, we determined whether the calgranulin homologue C (Cal C), which is expressed in cornea and neutrophilic granulocytes, is also detectable in psoriasis lesions. Therefore, mRNA was analyzed from shave biopsies of lesional and nonlesional psoriasis patients and healthy volunteers. Northern blot experiments demonstrated the strong expression in lesional, but not in nonlesional psoriatic skin. Normal keratinocytes cultured *in vitro* showed constitutive expression of Cal C and a decrease upon cultivation in the presence of serum (psoriatic and normal). However, on immunohistology using a rat polyclonal antiserum against Cal C, we were not able to detect Cal C in keratinocytes on a protein level (1 of 28 biopsies tested). Therefore, we speculate about the stability of the Cal C mRNA in keratinocytes, which needs to be determined. In conclusion, Cal C—another calcium-binding protein with potential relevance in the pathogenesis of psoriasis—is expressed in lesional skin at levels, that are substantially higher than expected by the presence of neutrophilic granulocytes.

## 743

**A Specific Multi-SNP Allele of HCR is Strongly Associated with Psoriasis in the Finnish Population**

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The authors present for the Finnish Psoriasis Consortium. In all populations, psoriasis associates strongly with the HLA-Cw6 allele, and the major role of this genomic region in psoriasis susceptibility has been confirmed by genetic linkage studies. However, the functional role of HLA-Cw6 has been questioned, and the association to it may be caused merely by another nearby gene. We focused our attention to a new gene called HCR (for alpha helical coiled coil rod protein) and found that a specific coding variant of HCR was strongly associated with psoriasis in a Finnish subpopulation (Asumalahti *et al*, *Hum Mol Genet* 9:1533–1542, 2000). We expanded this study by recruiting further families (*N* = 91) from other parts of Finland, representing a more general population sample. 78% of all the patients had plaque type disease and 101 patients had type I psoriasis. The median age at onset was 20 years, male:female ratio was 69:47, and 34% of probands had an affected first-degree relative. 18% of patients and 7% of controls carried the HLA-Cw6 allele (*p* = 0.005). The HCR risk allele consisted of two SNPs encoding for Arg to Trp changes; we call the risk allele HCR\*WW. HCR\*WW was present in 27% of patients and in 10% of controls (*p* = 0.0001). The suggested corneodesmosin (S gene) risk allele CD\*5 was the predominant allele in control as well as patient chromosomes, providing further evidence against it as the susceptibility allele. These results were supported by the transmission disequilibrium test. We conclude that HCR is a strong candidate for the psoriasis susceptibility locus PSORS1.

## 740

**Improvements to MelanoDerm™, An Epidermal Model Containing Functional Melanocytes For Skin Lightening Studies**

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Clinical skin lightening studies often run in excess of 3 months duration and are very costly. Therefore, there is considerable interest in developing methodology using cultured skin tissues to perform such studies *in vitro*. Towards this end, we have produced MelanoDerm, a highly differentiated, three-dimensional tissue culture model of human epidermis that contains normal human melanocytes (NHM) and keratinocytes (NHK). Cultures have been produced containing NHM of varying skin phototypes and pigmentation levels of the tissues follow the expected order, i.e. BlackAsianCaucasian. One previous limitation in utilizing such tissues has been the gradual decline in the tissue morphology. Over a 3-week period, tissues maintained in medium containing b-FGF and a-MSH (LLMM) gradually decrease in the number of viable cell layers (basal to granular layer, inclusive). Also, melanocytes with tissue maintained in LLMM have a tendency to clump and lose dendricity over this time period. To address these drawbacks, a new maintenance medium (NMM) has been developed which omits stimulators of melanogenesis yet maintains epidermal structure (almost without any decline) over periods of at least 4 weeks. In addition, melanocytes within the tissue remain distinct and highly dendritic. Quantitative melanin assay results show that melanin levels in tissues grown in LLMM and NMM are equivalent. These results demonstrate that MelanoDerm cultured in NMM will be useful for extended studies of melanogenesis and skin lightening.

## 742

**Identification and Characterisation of Corneodesmosin Single Nucleotide Polymorphisms**

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Psoriasis is a chronic inflammatory disorder that affects approximately 2% of the population of European ancestry. Family based studies located a major susceptibility locus (PSORS1) within the MHC on chromosome 6p21.3. The most significant association has been shown with HLA-Cw6 in psoriatic patients with a strong family history. However, recent attention has been focussed on non-HLA genes close to HLA-C, in particular the corneodesmosin (CDSN) gene, located approximately 160kb telomeric of the HLA-C locus. The CDSN gene contains 2 exons and spans 5.6 kb. Corneodesmosin is expressed during keratinocyte differentiation and has been shown to be over-expressed in psoriatic lesions compared with normal skin. We have reported significant association between a polymorphism at position 1243 of the corneodesmosin gene and psoriasis (Tazi-Ahnini *et al* 1999). To further define the role of corneodesmosin in the genetic predisposition to psoriasis, we have exhaustively screened the corneodesmosin gene for single nucleotide polymorphisms (SNPs). Genomic DNA from a panel of 24 unrelated individuals was amplified with oligonucleotide primers spanning 350bp 5' UTR, exon1, exon 2 and 910bp 3' UTR. A total of 37 SNPs were identified by direct DNA sequencing. These include 35 SNPs and 2 insertion/deletion polymorphisms. 18 polymorphisms are present within exons 1 and 2; 9 are neutral polymorphisms, 8 result in nonconservative amino acid substitutions and 1 an amino acid insertion/deletion polymorphism. Assays have been developed (RFLP and single base extension) and validated for all polymorphisms, and allele frequencies determined in a panel of 96 unrelated blood donor controls. These data are being used to define the pattern of linkage disequilibrium between SNPs within the CDSN gene. This will provide a comprehensive panel of SNPs for the analysis of haplotype transmission in psoriasis families.

## 744

**Sequence Variation in a Retrovirus-Like Expressed Sequence Tag in the PSORS1 Critical Region of the MHC: Lack of Evidence for a Causative Psoriasis Gene**

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Several genome-wide searches have shown that an important locus for psoriasis susceptibility resides in the major histocompatibility complex on chromosome 6p21.3. We mapped this locus (PSORS1) to a 60-kb interval extending from 25 to 85 kb telomeric of HLA-C by recombinant ancestral haplotype analysis. In psoriatic individuals, eight microsatellite markers across the 60 kb region yielded an identical haplotype on all risk chromosomes, which was absent from all nonrisk chromosomes. This risk haplotype, called RH1, defines a genetic interval containing at least 9 expressed sequence tags (ESTs). Five of these ESTs are expressed in skin, as determined by PCR analysis of normal and psoriatic skin-derived cDNA libraries. We completely sequenced one of the skin-expressed ESTs (which we designated A119) and found that its entire length (2.6 kb) is represented in the genomic sequence of RH1 (99% identity). A119 sequences demonstrate sequence similarity to retroviral integrase and envelope glycoproteins, identifying it as a human endogenous retrovirus-like element. In an effort to identify disease-specific polymorphisms in A119, we examined its DNA sequence across 5 risk and 7 nonrisk haplotypes. The A119 region was PCR amplified from genomic DNA, cloned in TOPO XL vector to separate alleles, and sequenced using primers within and flanking A119. We identified 27 polymorphisms in the A119 sequence, including 26 single-nucleotide polymorphisms (SNPs) and one two-base indel. None of the observed variations were specific for RH1. These results indicate that A119 is unlikely to play a causative role in psoriasis.

## 745

**Analysis of HCR Gene Polymorphism in Familial Psoriasis: Evidence Against a Causal Role**

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A recent report identified 12 coding variations within the HCR (Pg8) gene located in the HLA class I region (*Hum Mol Genet* 9:1533–1542). Two of these variations, C→T transitions at nucleotide positions 251 and 269, form an allele (the “TT” allele) that was suggested to associate with psoriasis. We had previously mapped the PSORS1 susceptibility locus to a 60-kb segment telomeric of HLA-C (*Am J Hum Genet* 66:1833–1844). Using 34 closely spaced microsatellite markers, we identified 66 relatively homogenous clusters of haplotypes in 478 families and examined their association with psoriasis by the transmission/disequilibrium test. We found that cluster numbers 17–25 conferred risk for psoriasis and possessed identical alleles at eight contiguous markers spanning the 60 kb interval, which we termed risk haplotype 1 (RH1). All risk clusters in our sample carried RH1, but none of the nonrisk clusters did. Due to the close proximity of RH1 to the HCR gene, we wished to determine whether the RH1-bearing risk haplotypes we identified carry the “TT” allele, and whether the nonrisk clusters do not. We PCR amplified exon 2 of the HCR gene from genomic DNA of 32 individuals representative of the seven most common risk and five most common nonrisk haplotypes in our sample. The gel-purified PCR products were directly sequenced for the region containing HCR nucleotides 251 and 269. The results showed that chromosomes bearing risk haplotypes 17 and 18 carry C at nucleotide 251 and 269, whereas risk clusters 19, 21, 22, 23 and all carry T at these positions. Among nonrisk clusters, cluster 26 carried T at both positions and all other clusters carried C. Since TT alleles are found in both risk and nonrisk clusters, we conclude that these variations in HCR are not likely to be causal in psoriasis.

## 747

**Polymorphism of Vitamin D Receptor Gene in Japanese Patients with Psoriasis Vulgaris**

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Vitamin D (VD) is known to be effective for psoriasis because it is an antiproliferative factor for several cell types such as keratinocytes and lymphocytes. There are some polymorphisms in vitamin D receptor (VDR) gene recognized by restriction enzymes such as FokI, BsmI and ApaI. Particularly, FokI polymorphism is demonstrated to be associated with the reactivity for VD in antiproliferative effect. In this study, we analyzed FokI polymorphism in patients with psoriasis vulgaris. We examined 96 unrelated Japanese patients with psoriasis vulgaris and 67 healthy Japanese persons were served as control subjects. Genomic DNA was extracted from peripheral blood leukocytes, and the exon 2 of VDR gene was amplified by PCR. Restriction patterns by FokI could distinguish F allele from f allele. FokI phenotype frequencies were compared between patients and controls. We also classified 30 psoriatic patients in terms of response to VD (tacalcitol) topical treatment into three groups: extremely responsive (n=6), moderately responsive (n=15) and barely responsive patients (n=9). FokI phenotype frequencies were also compared among these groups. FF, Ff and ff frequencies are as follows: FF=31%, Ff=50%, ff=19% in patients; FF=43%, Ff=43%, ff=13% in controls; FF=66%, Ff=17%, ff=17% in extremely responsive patients; FF=33%, Ff=40%, ff=27% in moderately responsive patients; FF=0%, Ff=67%, ff=33% in barely responsive patients. FF phenotype tended to be decreased in patients compared with controls (31% vs. 43%), which was not statistically significant. FF phenotype was significantly increased in extremely responsive patients compared with barely responsive patients (66% vs. 0%, p<0.05). Although it is necessary to reanalyze FokI phenotype frequencies among larger number of psoriatic patients, this result is intriguing because F allele is demonstrated to be more responsive to VD *in vitro* compared with f allele.

## 749

**Compound Heterozygosity for a Recurrent 16.5-kb Alu-Mediated Deletion Mutation and Single-Base Pair Substitutions in the ABCC6 Gene Results in Pseudoxanthoma Elasticum**

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Pseudoxanthoma elasticum (PXE), the prototypic heritable connective tissue disorder affecting the elastic structures in the body, manifests with cutaneous, ophthalmologic and cardiovascular findings, with considerable morbidity and mortality. Recently, mutations in the ABCC6 gene encoding MRP6, a putative transmembrane ABC transporter protein of unknown function, have been disclosed, and most of the genetic lesions delineated thus far consist of single-base pair substitutions resulting in nonsense, missense or splice-site mutations. In this study, we examined four multiplex families with PXE inherited in an autosomal recessive pattern. In each family, the proband was a compound heterozygote for a single-base pair substitution mutation, resulting either in nonsense or splicing mutations, and a novel, ~16.5-kb deletion mutation spanning the site of the single-base pair substitution *in trans*. The deletion mutation was shown to extend from intron 22 to intron 29, resulting in out-of-frame deletion of 1213 nucleotides from the corresponding mRNA, and causing a predicted elimination of 505 amino acids from the MRP6 polypeptide. The deletion breakpoints were precisely the same in all four families. Distinct ethnic backgrounds and haplotype analysis by 13 microsatellite markers spanning 9 cM of the PXE locus confirmed that the deletion had occurred independently. Deletion breakpoints within introns 22 and 29 were embedded in two highly homologous AluSx repeat sequences, suggesting Alu-mediated homologous recombination as a mechanism.

## 746

**Differential Relationship of Disease Severity to Age of Onset in Familial vs. Sporadic Psoriasis**

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To evaluate the relationship between psoriasis disease severity, age of onset, and family history, 537 psoriatics were analyzed, most of whom were from Michigan. In 227 individuals there was no known family history, and in 310 individuals psoriasis was reported or demonstrated in at least one first-degree relative. 98% of probands were ascertained for early onset psoriasis (<40 year at onset); therefore, late-onset psoriatics were under-represented. Total body surface area involvement (TBSA), presence or absence of joint complaints (with or without visible joint changes) and nail changes (pitting, oil spots, or onycholysis) served as measures of disease severity. Two-way ANOVA after log transformation revealed highly significant interaction between age at onset and familial status (p=0.00001). Two-sample t tests showed that for sporadic psoriatics, TBSA was significantly less when onset was early (14.3% vs. 28.0%, p=0.0034), whereas for familial psoriatics, TBSA was significantly greater when onset was early (15.1% vs. 8.7%, p=0.00003). Using loglinear analysis, arthritis and age at onset were not significantly associated after controlling for age at examination, nor were arthritis and familial status. By the same method, nail abnormalities were also conditionally independent of familial status, given age at onset. However, psoriatic nail changes were more frequently encountered in early onset patients. These results suggest that the relationship between disease severity and age at onset differs in sporadic vs. familial psoriasis. This finding may reflect the concurrent action of psoriasis-predisposing alleles at multiple loci in “familial” cases, whereas environmental factors may play a larger (but not the only) causative role in “sporadic” cases.

## 748

**The Polymorphism of Vitamin D<sub>3</sub> Receptor Gene in Patients with Psoriasis**

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Vitamin D<sub>3</sub> is an effective drug for the treatment of skin eruptions in patients with psoriasis. It has been reported that vitamin D receptor gene show considerable polymorphism. In 86 normal subjects and 39 patients with psoriasis, the allelic frequencies of the vitamin D receptor and the possible association of vitamin D<sub>3</sub> receptor gene polymorphism and clinico-laboratory findings were studied. Using heminested polymerase chain reaction, 3 sites of vitamin D receptor gene containing the polymorphism in the genome DNA purified from blood were amplified. The polymorphism was studied using restriction pattern of the polymerase chain reaction products. No significant difference between normal controls and patients with psoriasis were found for the frequencies of 3 kinds of alleles. In patients with psoriasis, serum alanine aminotransferase of AA allele were significantly higher than that of aa allele (p<0.05). Serum triglyceride in AA allele were significantly higher than those in Aa allele (p<0.05) and aa allele (p<0.02). No other clinico-laboratory findings showed relation to vitamin D receptor gene polymorphism. These results showed that polymorphism at intron 8 of vitamin D receptor gene has relation to metabolic abnormalities including liver dysfunction and hyperlipidemia in patients with psoriasis.

## 750

**A Spectrum of ABCC6 (MRP6) Mutations are Responsible for Pseudoxanthoma Elasticum**

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Pseudoxanthoma elasticum (PXE) is a heritable disorder characterized by calcification of elastic fibers in skin, arteries and the retina, that results in dermal lesions with associated laxity and loss of elasticity, arterial insufficiency and retinal hemorrhages leading to macular degeneration. PXE is usually found as a sporadic disorder but examples of both autosomal recessive and dominant forms of PXE have been observed. Recently, dominant and recessive forms of PXE were mapped to a 5-cM domain on chromosome 16p13.1. We have refined this locus by recombination mapping and identified six candidate genes. This locus was further reduced to less than 600 kb and five candidate genes. These candidate genes were screened for mutations using genomic DNA from a cohort of unrelated patients with autosomal recessive or sporadic PXE. We have found causative mutations in a gene encoding an ATP-binding cassette (ABC) transporter referred to as ABCC6 (MRP6). ABCC6 belongs to a subfamily of genes that includes the Cystic Fibrosis Transmembrane conductance Regulator gene (CFTR). Although most of the variants identified in the ABCC6 gene, thus far, were single-nucleotide substitutions resulting in missense mutations, we have also observed nonsense, splice site mutations as well as insertions and deletions. The majority of the mutations appeared to be private. However, a few recurrent mutations were found in unrelated families of different origins with distinct haplotypes. ABCC6 mutations were primarily located in the 3' end of the gene particularly in domains encoding the C-terminal ATP-binding site where 40% of the variants were clustered. Remarkably, missense mutations affected essentially arginyl residues suggesting an essential structural or functional role for these residues in ABCC6.

## 751

### Characterization of ABCC6 Pseudogenes on Human Chromosome 16p: Implications for Mutation Detection in Pseudoxanthoma Elasticum

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Pseudoxanthoma elasticum (PXE) is a heritable disorder affecting the skin, eyes and the cardiovascular system, and recently, it has been linked to mutations in the ABCC6 gene on chromosome 16p13.1. Our original mutation detection strategy, consisting of amplification of each exon of the ABCC6 gene by the use of primer pairs placed on the flanking introns, suggested the presence of multiple copies of the 5'-region sequences of the gene. By the use of allele specific PCR, two BAC-clones containing a putative pseudogene of ABCC6, designated as ABCC6P1, were isolated from the human BAC-library. Sequence analysis of ABCC6P1 revealed several single-base pair nucleotide changes in regions homologous to coding as well as intronic sequences of ABCC6. ABCC6P1 was shown to be a truncated copy of ABCC6 containing exons 1–9 and the upstream region of the gene. Radiation hybrid and marker content mapping placed this pseudogene 280 kb centromeric from the functional ABCC6 gene. In addition to ABCC6P1, homology search of the database of Human Genome Working Draft revealed the presence of another truncated copy of ABCC6, designated as ABCC6P2, which was shown to harbor upstream sequences and exons 1–4. The BAC-clone containing ABCC6P2 was placed ~1.1 Mb telomeric from the BAC-clone containing ABCC6. In addition to several nucleotide differences in flanking introns, ABCC6P2 contained two nucleotide changes in the sequences homologous to exons 3 and 4 of ABCC6. Nucleotide differences in flanking introns between these two pseudogenes and ABCC6 allowed us to design allele specific primers which eliminate the presence of both pseudogene sequences in PCR products, providing reliable amplification of the ABCC6-specific sequences only. The use of allele specific PCR has revealed thus far one pathogenetic PXE mutation, 179del9 in exon 2, as well as several polymorphisms within the upstream region and exons 1–9.

## 753

### In Contrast to Patients with the Type VIA Variant of Ehlers Danlos Syndrome, a Decrease in One of the Isoforms of Lysyl Hydroxylase Does Not Appear to be a Common Cause of the Type VIB Variant

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Lysyl hydroxylase 1 (LH1) is an enzyme required for the hydroxylation of specific lysine residues in the collagen molecule. These hydroxylsines are critical in determining the pathways of intermolecular crosslinks that are essential for the tensile strength of collagen. LH1 is deficient in patients with Ehlers Danlos syndrome (EDS) type VIA, an autosomal recessive disorder in which patients are clinically characterized by kyphoscoliosis, hypermobile joints, and skin that is soft, fragile and hyperextensible. In a second class of EDS VI, the type B variant, patients have the clinical phenotype of EDS VI but their levels of LH activity are not significantly decreased. To examine the biochemical basis for this form of EDS VI, we compared levels of the mRNAs for LH1 and the two other recently reported isoforms for LH, LH2 and LH3, with the pattern of crosslinking, and enzyme activity in fibroblasts from 8 patients with EDS VIB. In contrast to EDS VIA patients, in which levels of LH1 mRNA and corresponding LH activity are < 25% of control, LH1 mRNA levels in EDS VIB patients ranged between 87 and 150% of control which correlated with their normal LH activity. However, decreased levels of LH2 mRNA were observed in two EDS VIB patients (25 and 50% of control, respectively), and in the first patient, the low (25%) level of LH2 mRNA was accompanied by a similar decrease (18% of control) in the mRNA for LH3. Another patient also had decreased levels of LH3 mRNA (39% of control). Studies on long-term cultures of fibroblasts did not show a correlation between levels of the collagen reducible crosslinks, dehydrodihydroxylysinoxidation and dehydrohydroxylysinoxidation, and levels of mRNA for the LH isoforms. This preliminary study suggests that, unless this group of patients represents a heterogeneous subset of the type VIB variant of EDS or an unidentified LH isoform is involved, some alternative pathway other than lysine hydroxylation of collagen is affected in these patients.

## 755

### A G to A Change at the Splice Donor Site of Intron 2 in the Xeroderma Pigmentosum Group C (XPC) Gene Alters the Efficiency of PremRNA Splicing

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We studied the molecular defects in the XPC gene in a 47 y old woman with XP (XP86BE) with severe sun sensitivity and multiple cutaneous neoplasms. UV cell killing was 3 times normal in XP86BE skin fibroblasts. Host cell reactivation of a UV-treated reporter plasmid cotransfected with a vector expressing wild type XPC message assigned XP86BE to XP complementation group C. RT-PCR of total RNA revealed two alternatively spliced XPC mRNA isoforms in the exon 2 region: (I) a deletion of 68 bases at the 3' end of exon 2 and (II) a 141 base insertion between exons 2 and 3 that retains the 5' end of intron 2. These changes resulted in frameshifts with new termination sites that would encode truncated XPC proteins. Genomic DNA analysis showed a homozygous G to A mutation at the splice donor site of XPC intron 2. This mutation reduces the information content of splice donor 2 from 10.2 to ~2.6 bits and results in use of cryptic donor sites of 8.6 bits (isoform I) and 9.5 bits (isoform II). The mutation inactivates a Bsu36 I site in intron 2 that can be used to diagnose the genomic status of other family members. The splice donor site mutation identified in XP86BE cells resulted in alternatively spliced XPC mRNA isoforms which utilize cryptic donors with high information content and are predicted to encode truncated XPC proteins with defective DNA repair function resulting in clinical disease.

## 752

### Two Commonly Occurring Mutations in the Lysyl Hydroxylase 1 Gene Decrease Enzyme Levels and are a Primary Cause of Ehlers–Danlos Syndrome Type VIA

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Patients with the autosomal recessive type VI variant of the Ehlers–Danlos syndromes (EDS VI: the kyphoscoliotic type), are clinically characterized by neonatal kyphoscoliosis, generalized joint laxity, skin fragility and severe muscle hypotonia at birth. Biochemically, EDS VIA is caused by a deficiency of lysyl hydroxylase (LH), an important post-translational modifying enzyme in collagen biosynthesis. This enzyme hydroxylates specific lysine residues in the collagen molecule to form hydroxylsines which have two important functions. The residues serve as attachment sites for galactose and glucosylgalactose and they also act as precursors for the crosslinking process that gives collagen its tensile strength. At least 20 different mutations have been identified in the LH1 gene (the originally described form) that contribute to LH deficiency and the clinical characteristics of EDS VI. Two of these mutations have been identified in five or more unrelated patients and appear to be the major cause of the enzyme deficiency that weakens the extracellular matrix and causes EDS VI in these patients. In one mutation there is a large duplication of exons 10–16, arising from a homologous recombination of intronic Alu sequences, and the second mutation is a nonsense mutation, Y511X, located in exon 14 of the LH1 gene. Each of these mutations appears to have originated from a single ancestral gene. Alternative processing pathways involving alternate splicing and mRNA degradation, which reduce the effect of the mutant allele containing the Y511X mutation, have been identified. This alternative processing has also been associated with other mutations in the LH1 gene and may represent an attempt to restore partial activity of the enzyme in these EDS VIA patients.

## 754

### Genetic Analysis of Kindler Syndrome in Panama

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Kindler Syndrome is a rare genodermatosis characterized by congenital acral bullae, progressive poikiloderma, photosensitivity, cigarette paper-like wrinkling of the dorsum of the hands and feet, diffuse cutaneous atrophy, webbing of the digits and easily bleeding buccal mucosa. No more than several dozen patients have previously been described in the literature. We are searching for the genetic basis of this rare photosensitivity disorder in an inbred group of twenty-six Kindler Syndrome patients who are members of the Ngobe Tribe from the province of Bocas del Toro, Panama. Additional findings included blisters induced by trauma and sun exposure in all patients, loss of the nailfold angle in eight patients, hypermobility of the fingers, knees and elbows in five patients and clinical features resembling ribbed epidermolytic hyperkeratosis of the lateral ankles in two patients. Six of the 11 male patients had phimosis. Most patients demonstrated significant periodontal attachment loss around their teeth. Because Kindler Syndrome has clinical features overlapping those of Rothmund-Thompson and Bloom Syndromes and these latter are caused by mutations in genes encoding RecQ helicases, we began by screening our patients for linkage of disease to regions harboring two other cloned RecQ genes. The results of this initial screen were negative. We have now proceeded to a genome-wide scan with 811 microsatellite repeat markers. Initial results show several promising areas.

## 756

### Influence of a Single Nucleotide Polymorphism in an XPC Splice Acceptor Site on Alternative Splicing

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Possible variations in DNA repair due to inherited polymorphisms of DNA repair genes are being investigated in order to determine their association with cancer predisposition. We previously found a common biallelic poly AT insertion/deletion polymorphism (PAT) in intron 9 of the XPC gene (*Carcinogenesis* 21:1821, 2000). Here we report a single nucleotide polymorphism (SNP) in XPC intron 11 acceptor (–6 A to C) that inactivates a Bcg I site. This SNP was genotyped in 97 normal donors by PCR followed by RFLP. The frequency of the A allele was 42% and the C allele was 58%. The A to C change increases the information content of the XPC intron 11 splice acceptor from 5.1 to 7.5 bits. When we analyzed the cDNA from normal individuals either homozygous (A/A or C/C) or heterozygous (A/C) for this SNP we found two XPC mRNA isoforms: (I) normal and (II) deletion of exon 12 resulting in a frameshift with termination signal 1 codon downstream. We have developed extremely sensitive, isoform specific, real time, quantitative RT-PCR assays to measure both isoforms. We found that cultured normal fibroblasts contain measurable levels of both isoforms. We are planning to determine if the level of the alternatively spliced isoform (II) is correlated with the XPC intron 11 SNP genotype. Interestingly, we found that the intron 11 SNP A allele is in linkage disequilibrium with PAT + (7.5 kb 5') and another SNP in exon 15 (C2920A, 2.5 kb 3'). An XPC intron 11 SNP-mediated effect on alternative splicing of XPC message might be a mechanistic link to cancer predisposition in association with the PAT + genotype.

## 757

**101 Prenatal Diagnoses by the DebRA Molecular Diagnostics Laboratory at Jefferson**  
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The DebRA Molecular Diagnostics Laboratory at Jefferson performed 101 prenatal diagnoses during the period January 1, 1993 to December 31, 2000 in families with a previously affected child with either the Junctional (JEB) or Dystrophic (DEB) forms of Epidermolysis Bullosa. Mutation studies were performed using heteroduplex analysis of PCR products spanning all exons of the appropriate gene for the diagnostic category, followed by direct genomic sequencing. Linkage studies were performed with intragenic and flanking SNP and/or microsatellite markers. Linked markers were used in conjunction with mutation studies for 7 JEB and 20 DEB cases while linked markers only were used for 17 DEB cases. In all, 51 JEB and 50 DEB prenatal diagnoses were performed with outcomes consistent with predicted phenotype in 60 cases, while in 31 cases outcome was not confirmed but laboratory error was not reported. Eight pregnancies are ongoing and there was one miscarriage. In one DEB case, submitted in 1994, where linked markers were used along with a single mutation, an affected fetus was predicted while normal fetal skin was found by concurrent EM studies. The cause of this discrepancy was never determined but the DNA result was confirmed. Possible explanations include a de novo mutation or germline mosaicism. All tests were performed for recessive forms of JEB and DEB, and predicted outcomes of 21 homozygous normal: 51 heterozygous carrier: 24 homozygous affected fetuses were reported for 96 pregnancies. These values are consistent with the expected corresponding Mendelian ratio of 1:2:1. There were 2 known recombinations in DEB linkage studies and 2 pregnancies where linked markers were inconclusive. Therefore, prenatal diagnosis for JEB and DEB using mutation studies was found to be 100% accurate and linked markers were found to be 99% accurate with 2 recombinations in 37 DEB cases.

## 759

**Phenotypic Severity of Epidermolysis Bullosa Simplex Caused by a Dominant Missense Mutation in the keratin 5 is Exacerbated When Combined with a Silent Missense Mutation in Another Allele**

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Epidermolysis bullosa simplex (EBS) is a group of hereditary skin disorders characterized intraepidermal cytolytic blister formation after mechanical trauma. Mutations in the either gene encoding keratin 5 and keratin 14, the primary structural components of the basal epidermal keratinocytes, lead to three major subtypes of EBS, including Weber-Cockayne, Koebner, and Dowling-Meara variants. In this study, we have investigated a Japanese EBS family whose members demonstrated heterogeneous clinical severity. A proband, 22-year-old man presented blisters on the whole body at childhood, and constantly forms blister by minor friction on the hands and feet at adulthood, while his father, his paternal uncle and grandmother showed a few blisters on the soles only after waking for a long time or putting on new shoes. To elucidate the molecular basis of this apparent clinical heterogeneity, we studied genomic DNA extracted from peripheral blood of the patient, his mother and paternal uncle, using PCR amplification followed by direct nucleotide sequencing. A novel missense mutation in the KRT5, E170K, at the conserved 1 A helix initiation site of the rod domain was found in the proband and his paternal uncle. Interestingly, another amino acid substitution E418K at the 2B rod domain in keratin 5 was identified in his clinically unaffected mother as well as in the proband. E418K was not found among the 100 normal Japanese alleles, thus considered to be silent missense mutation, not polymorphism. As shown in the proband, these results suggest that mild clinical phenotype of Weber-Cockayne EBS due to E170K is aggravated when combined with a silent E418K mutation in another allele of KRT5. This is the first example of keratin disorders in which clinical severity caused by a dominant missense mutation is shown to be exacerbated by the presence of a silent missense mutation in another allele.

## 761

**The Gene for Epidermolysis Bullosa Simplex Superficialis Maps to Chromosome 3p21**  
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Epidermolysis bullosa simplex (EBS) is an inherited blistering disease characterized by intraepidermal cleavage. In a particular and very rare subset of EBS described by Fine *et al* (1989), known as "EBS superficialis", blistering and cleavage occurs just beneath the stratum corneum. This unique presentation of EBS has been reported in only two unrelated families, emphasizing the uniqueness of these findings. In order to identify the gene responsible for EBS superficialis, we performed a genome-wide screen in one of the families, a dominant pedigree with 25 affected individuals. Using a panel of 324 fluorescently labeled microsatellite markers, distributed at an average of 10-cM intervals, we established linkage ( $Z_{max}=3.72$ ) to a 3.47-cM region on chromosome 3p21. The type VII collagen gene lies within this linkage interval, and although an unlikely candidate gene for this type of EB, it was excluded by heteroduplex analysis and direct sequencing. We are currently undertaking studies to narrow this interval to identify putative candidate genes. The discovery of the gene underlying EBS superficialis will provide further understanding of the mechanisms controlling the final steps in the differentiation of the epidermis and extend our knowledge of the molecular basis of the different forms of EB.

## 758

**Paternal Germline Mosaicism in Herlitz Junctional Epidermolysis Bullosa**

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We studied a single patient with the lethal (Herlitz) type of junctional epidermolysis bullosa (H-JEB) born from unrelated parents. Screening for mutations in the LAMB3 gene in the patient revealed the previously described hotspot mutation R635X and a novel one basepair deletion in exon 10 (1094delA). The single basepair deletion could be detected in the clinically unaffected mother, while the nonsense mutation R635X could not be found in the peripheral blood DNA of either parent. After excluding nonpaternity by microsatellite analysis using random markers on chromosomes 3, 8 and 18, we determined that the mutation R635X in the proband was most likely due to a de novo event or alternatively, germline mosaicism. The parents requested prenatal diagnosis for a second pregnancy, and while the maternal mutation 1094delA was not detected in DNA from the fetus, unexpectedly, the mutation R635X was present in the chorionic villus DNA. These findings were most consistent with paternal germline mosaicism for the recessive mutation R635X. The results have a significant impact on genetic counseling in this family. To our knowledge, this study represents the first documented case of germline mosaicism in junctional epidermolysis bullosa and serves as a reminder that germline mosaicism should be considered in every case in which a "new" mutation is found in the offspring of clinically and/or genetically unaffected parents.

## 760

**Maternal Germline Mosaicism in Dominant Dystrophic Epidermolysis Bullosa**

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We studied a small pedigree in which one child was affected with the mild form of dystrophic epidermolysis bullosa (DEB). Screening for mutations in the COL7A1 gene in the patient revealed a single glycine substitution in the triple helical domain of the type VII collagen gene (G2003R), which has previously been associated with Bart's syndrome, a form of dominantly inherited DEB. This mutation, however, could not be found in the peripheral blood DNA of the clinically unaffected parents, thereby suggesting a *de novo* mutation or alternatively, germline mosaicism. The parents requested prenatal diagnosis for a third pregnancy, and unexpectedly, the mutation was also present in the chorionic villus DNA from the fetus, consistent with germline mosaicism. Microsatellite analysis revealed that all three offspring carried the same maternal chromosome 3 haplotype, while the two offspring carrying the mutation G2003R had inherited different paternal chromosome 3 haplotypes. These findings were consistent with maternal germline mosaicism for the COL7A1 mutation G2003R. The results have a significant impact on genetic counseling in this family. We believe this study represents the first documented case of germline mosaicism in DDEB. Germline mosaicism should be considered in every case in which a "*de novo*" mutation is found in the offspring of unaffected parents.

## 762

**A Heterozygous Deletion Mutation of Keratin 5, Resulting in the First Case of a Longish Mutant Keratin, in an Atypical Epidermolysis Bullosa Simplex Case**

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Epidermolysis bullosa simplex (EBS) is a dominantly inherited blistering skin disease caused in most cases by point mutations in keratin (K) 5 and 14 genes. Two deletion mutations of K14 which resulted in truncated keratins have been previously reported in autosomal recessively EBS with severe clinical phenotypes, and also a deletion mutation of K14 in a case of the Weber-Cockayne type EBS has been reported. Here, we have found the first case of deletion mutation of K5, a single nucleotide heterozygous deletion, resulting in a longish mutant keratin, in a Japanese child with atypical EBS. The patient had an unusual phenotype as EBS, showing multiple vesicles in an annular configuration at the edge of migratory multiple erythema, ranging from coin- to head-size in diameter, on the trunk and legs. Electron microscopy of skin biopsies showed a marked reduction in number of keratin intermediate filaments in the basal cells without tonofilament clumping. Immunofluorescence microscopy showed no or faint staining in basal cells by using monoclonal antibody C-50 and RCK102 against K5/8. A heterozygous deletion of G from the second base position of amino acid codon 550 (V2 domain) of K5 was found by sequence analysis of genomic DNA. This deletion caused a frameshift, which leads to a readthrough mutation, creating a terminal codon in the end domain of K5. The position of this terminal codon was located in the furthest downstream among the reported cases suggesting the generation of the first case of the longish mutant K5. This abnormally longish mutant keratin protein reasonably appears to exert a dominant negative effect on the keratin intermediate filament cytoskeleton in basal cells, leading to an atypical clinical phenotype as the present case, which has never been reported.

## 763

**Evidence for Novel Functions of the Keratin Tail Emerging from a Mutation Causing Ichthyosis Hystrix**

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Unraveling the molecular basis of inherited disorders of epithelial fragility has led to understanding of the complex structure and function of keratin intermediate filaments (KIF). Keratins are organized as a central alpha-helical rod domain flanked by nonhelical, variable end domains. Pathogenic mutations in 19 different keratin genes have been identified in sequences corresponding to conserved regions at the beginning and end of the rod. These areas have been recognized as zones of overlap between aligned keratin proteins and are thought to be crucial for proper assembly of KIF. Consequently, all keratin disorders of skin, hair, nail and mucous membranes caused by mutations in rod domain sequences are characterized by perinuclear clumping of fragmented KIF, thus compromising mechanical strength and cell integrity. We report here the first mutation in a keratin gene (KRT1) that affects the variable tail domain (V2) and results in a profoundly different abnormality of the cytoskeletal architecture leading to a severe form of epidermal hyperkeratosis known as ichthyosis hystrix Curth-Macklin. Structural analyses disclosed a failure in KIF bundling, retraction of the cytoskeleton from the nucleus, and failed translocation of Iorocrin to the desmosomal plaques. These data provide the first *in vivo* evidence for the crucial role of a keratin tail domain in supramolecular KIF organization and barrier formation.

## 765

**Focal Palmoplantar and Gingival Keratosis is Characterized by Epidermolytic Alterations and Keratin 16 Gene Mutations**

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Focal palmoplantar and gingival keratosis (FPGK), firstly by defined Gorlin in 1976, is a rare autosomal dominant palmoplantar keratoderma with oral leukoplakia (MIM \*148730) that shows some clinical overlap to pachyonychia congenita type I. The light and electron microscopical alterations and keratin mutations of FPGK are currently not known. We recently observed a family affected by the disease in at least three consecutive generations. The four patients examined suffered since infancy from circumscribed and sometimes painful hyperkeratosis at the weight-bearing plantar skin, rather mild palmar hyperkeratosis, and continuous leukoplakic lesions confined to the maxillary and mandibular attached gingiva. There were no follicular hyperkeratosis and no nail thickening, the diagnostic hallmark of pachyonychia congenita type I. Light and electron microscopy revealed epidermolytic alterations in both the suprabasal epidermis and epithelium, with edematous keratinocytes, abnormal clumping of tonofilaments, and irregularly shaped keratohyaline. These alterations were confined to nests of keratinocytes in the plantar lesions, but were pronounced in the gingival hyperkeratosis. Immunohistochemistry failed to show regular expression of keratin 16, and linkage analysis of K6 and K16 genes demonstrated mutations in keratin 16. We conclude that FPGK is a clinically and pathologically distinct epidermolytic palmoplantar keratoderma caused by keratin 16 mutations. The K16 mutations are obviously less disruptive than in pachyonychia congenita type I that is characterized by more pronounced molecular and pathological alterations of the tonofilament organization with epidermolytic blistering.

## 767

**Novel ATP2A2 Gene Mutations in Two Chinese Pedigrees with Darier's Disease**

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Darier's disease (DD; keratosis follicularis) is an autosomal dominant skin disorder characterized by warty papules with distribution over seborrheic areas. Recently, the cause of DD was identified to be due to mutations in the ATP2A2 gene on 12q23–24.1, encoding the sarco-endoplasmic reticulum calcium ATPase type 2 (SERCA2), a Ca<sup>2+</sup> pump that plays an important role in intracellular Ca<sup>2+</sup> signalling. Here we report two novel point mutations in the ATP2A2 gene in two Chinese families with DD. Genomic DNA was extracted and each of the 21 exons of the ATP2A2 gene was amplified by PCR. Amplified DNA fragments were analyzed using SSCP (single strand conformational polymorphism analysis) to look for abnormal gel migration. Fragments showing altered migration patterns were sequenced bi-directionally. In Family A, the extra band in exon 5 was sequenced and revealed a heterozygous point mutation of G to A at position 445 in exon 5 of the APT2A gene. This mutation is expected to cause replacement of the normal aspartic acid by asparagine (D149N) in the resultant protein. The proband from family B was found to have abnormal migration of fragments derived from exon 19. Sequencing of the extra exon 19 band showed a heterozygous point mutation of C to A at position 2747 in exon19, which changes codon 916 from serine to tyrosine (S916Y) in the resultant protein. These abnormalities were not found in any of the unaffected family members or in 50 unrelated healthy individuals. The missense mutations we describe here, D149N and S916Y, have not been described previously. They affect regions of the protein that encode IA strand and transmembrane domains, which have been shown to be highly conserved during evolution, as all known SERCA2 proteins from different species exhibit 100% identity in these domains. The D149N and S916Y mutations may alter the function of these important SERCA2 domains and, as a consequence, impair cell adhesion and differentiation of epidermis.

## 764

**A Novel and a Recurrent Mutation in Keratin 10 Causing Bullous Congenital Ichthyosiform Erythroderma**

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Bullous congenital ichthyosiform erythroderma (BCIE) is a congenital, autosomal dominant skin disease characterized by blister formation in neonates and generalized hyperkeratosis. Point mutations at the gene encoding keratins 1 (K1) and 10 (K10) have been identified in patients with BCIE and most of them had amino acid substitution in the highly conserved region of helix initiation motif of the keratins. Using direct sequencing of genomic DNA amplified by polymerase chain reaction, we have analyzed two sporadic Japanese patients with BCIE diagnosed clinically, pathologically, and ultrastructurally. The first case, a newborn male showing typical disease phenotype was found to have a single base pair substitution (CGC to CTC) at codon 156 of K10, resulted in an amino acid exchange from arginine to leucine (R156L). The second case, a 22-year-old male, with mild hyperkeratosis on the whole body revealed to have a novel single base pair substitution (TAC to TGC) at codon 160 of K10 resulted in an amino acid exchange from tyrosine to cysteine (Y160C). Both mutations were localized within the highly conserved helix initiation motif of K10 and excluded in a control population. Our present results were consistent with previous reports that the codon 156 is a mutation hot spot and that any mutation within the highly conserved helix initiation motif can lead to the disease. The tyrosine at codon 160 forms a hydrophobic core during heterodimer formation of keratin filaments and its substitution to hydrophilic amino acids (Y160N, Y160D and Y160S) has been reported to be associated with a very severe disease phenotype, although the severe phenotype was not observed in our case with Y160C mutation. Further accumulation of the cases should be required for elucidation of the genotype-phenotype correlation in the missense mutations of the tyrosine at the codon 160.

## 766

**Analysis of ATP2C1 Gene Mutations in 10 Unrelated Japanese Families with Hailey-Hailey Disease**

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Hailey-Hailey disease (HHD) is an autosomal dominant chronic blistering disease caused by a mutation in the ATP2C1 gene encoding P-type Ca<sup>2+</sup>-transport ATPase. In this study, we have examined the ATP2C1 gene in 10 unrelated Japanese families with HHD, and found five attributable mutations including four novel mutations, which include two missense mutations (C490F and L584P), one deletion (2460delG), and one splice-site mutation (1259+1 g to a). We could not detect any ATP2C1 mutations in five out of the 10 HHD families after direct sequencing of the entire coding region of ATP2C1 genomic DNA, including the exon-intron boundaries. Patients carrying missense mutations or a nonsense mutation in the 3' distal exon have tendencies of earlier disease onset compared to those with nonsense mutations in the 5' proximal exons. Our data provides a significant addition to the HHD mutation database and will contribute further to the understanding of HHD genotype/phenotype correlations.

## 768

**Identification of the Locus for a Novel Acantholytic Disease on Chromosome 12**

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We describe a family with a novel acantholytic disease, which although reminiscent of Darier-White and Hailey-Hailey diseases, appears to be a unique clinical variant. The characteristic rash does not begin in childhood, but after puberty or in early adult life, and there is a high degree of variability within the family, ranging from a generalized rash spread over the entire trunk, to a very limited distribution restricted to the distal extremities. The eruption is influenced by several factors, including temperature and humidity, and a skin biopsy showed some foci of acantholysis. The patients belong to a five-generation autosomal dominant pedigree originating from the Midwestern United States, with at least 20 affected members. Initially, we performed segregation analysis with markers near both the Darier-White and the Hailey-Hailey disease loci to exclude these genes. Subsequently, we performed a genome-wide scan with a panel of 324 microsatellite markers. Age-dependent penetrance was considered for the LOD score calculations. As a result, we have established linkage (Z<sub>max</sub>=4.3) to chromosome 12, in a region 25 cM proximal to the Darier-White gene (ATP2A2). Our future work is aimed to the fine mapping of the linked region and the characterization of possible candidate genes, based on the information available for Darier-White and Hailey-Hailey diseases.

## 769

**Increase of Transglutaminase 1 Expression by Interferon- $\gamma$  is Directly Mediated by STATs in Keratinocytes and Muscle Cells**

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The physiological significance of transglutaminase 1 (TGase 1) regulation by cytokine molecules is not determined yet although the increase of TGase 1 expression is responsible for hyperkeratosis in psoriatic lesions and squamous differentiation in squamous cell carcinomas. Recently, we discovered that TGase 1 expression is abundantly increased in sporadic inclusion body myositis (SIBM), a degenerative muscle disease. Increased inflammatory cytokines are detected in the lesion of SIBM since CD8+ T cells were infiltrated into the lesion. Furthermore, we found that mRNA of TGase 1 was increased 15 fold within 24 h by 1000 unit per ml INF- $\gamma$  treatment in the cultured myotubes. The major signal transducer in INF- $\gamma$  mediated gene expression is STAT (signal transducers and activators of transcription). Therefore we investigated the possible STAT-binding site (INF- $\gamma$  activation site, GAS) in the human TGase 1 promoter region. The DNA construct (1.3 kb) including the promoter region (-530), exon 1, and part of intron 1 of human TGase 1 was cloned into pCAT3 vector since it contains putative GAS. The construct was transfected into primary human keratinocytes (NHEK) and skeletal muscle cells (SkMC). After transfection and INF- $\gamma$  treatment, CAT activity showed 15 and 200% increase over the control in NHEK and SkMC, respectively. Gel shift data showed a specific DNA protein complex was formed by the oligo containing the GAS. In summary, we identified that TGase 1 expression may be directly modulated through the JAK-STAT signaling pathway in the inflammatory diseases.

## 770

**Homozygous Novel Deletion Mutation 1349delA in TGM1 Leads to a Classic Phenotype of Lamellar Ichthyosis (LI)**

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Malformation of the cornified cell envelope (CCE) arising from mutations of the transglutaminase (TGase) 1 gene (TGM1) is the cause of some cases of LI. However, genotype/phenotype correlation in TGM1 mutations has not been fully clarified yet. We report a typical case of LI caused by novel mutation in TGM1. The patient, a 33-year-old female, showed lamellar scales on the entire body surface. The patient's parents were distant relatives. Immunofluorescence labeling with anti-TGase1 antibodies was negative in patient's epidermis. *In situ* TGase activity assay detected markedly reduced TGase activity in granular layers of the patient's epidermis. Electron microscopy revealed incomplete thickening of CCE during keratinization in the epidermis. Sequencing of the entire exons and exon-intron borders of TGM1 revealed that the patient was a homozygote for a novel deletion mutation, 1349delA in exon 3. This mutation leads to frameshift resulting in a premature termination 43 bp downstream from the mutation site. According to the protein modeling of TGase 1, the truncated protein from this mutated allele loses entire catalytic core domain of TGase 1. Thus, the present homozygous mutation is expected to cause loss of TGase 1 activity resulting in a classic phenotype of LI in this patient.

## 771

**The *ichq* Mouse, a Model for Human Harlequin Ichthyosis: Mapping and Consideration of Candidate Genes Involved in Epidermal Differentiation**

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Harlequin ichthyosis (HI) is a rare and usually fatal scaling skin disorder. Because of the severity and trauma of the disorder, most families are small and genetic studies are difficult. The HI mouse *ichq/ichq* has many similarities to the human disorder and provides an important model to identify candidate genes. Here we report improved mapping of the *ichq* locus and consideration of candidate genes: *Capn1* encoding calpain 1, and *Rela* and *Ikka/Chuk* encoding components of the NF- $\kappa$ B pathway. These candidates were selected because calpain 1 expression is decreased in human HI, and alteration of the NF- $\kappa$ B pathway via IKK $\alpha$  has profound effects on skin differentiation. All candidates are close to the *ichq* locus on mouse chromosome 19, although *Ikka* is located more distally on Chr. 19. An intersubspecific cross was set up between BALB/cJ+/*ichq* and CAST/Eij, a wild inbred strain. The F1 progeny were then intercrossed and the F2 progeny were typed for the *ichq* phenotype. Linkage between *ichq* and molecular markers was calculated. Keratinocytes from skin of individual mice were cultured on collagen IV coated dishes as previously described, and tail DNA was extracted for genotype identification. Total RNA was extracted from keratinocytes of each genotype and used for RT-PCR amplification and sequence analysis for each candidate gene. *Capn1* was amplified in three overlapping segments. No mutations that resulted in a missense or frameshift were detected. Similar analysis of *Rela* and *Ikka/Chuk* also showed no such mutations. Analysis of CAPN1 cDNA from human HI cases revealed an R433P change, but analysis of 50 normal samples showed that this was a polymorphism, not a causative mutation. The results eliminate these three genes as the mouse *ichq* locus. Additional candidate genes found in the region of interest on mouse Chr. 19 and syntenic region of human Chr. 11 are being analyzed.

## 772

**Consequences of Dominant GJB3 Mutations on Gap Junction Communication in HeLa Cells**

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Recently we established the pathogenic role of heterozygous mutations in the connexin-31 (Cx31) gene (*GJB3*) in erythrokeratoderma variabilis (EKV), an autosomal dominant disorder of keratinization. To investigate the pathomechanisms and functional consequences of GJB3 mutations on Cx biogenesis and gap junction intercellular communication, we studied two missense mutations of different biological relevance in a mammalian expression system. G12R associated with EKV affects a conserved residue in the cytoplasmic amino terminus of Cx31, while R32W located in the first transmembrane domain is not disease-causing in a heterozygous state. Communication-deficient HeLa cells were stably transfected with wildtype and mutant Cx31. Using immunostaining, both mutant proteins were detected at the plasma membrane in a punctate pattern similar to wildtype Cx31, indicating that synthesis, transport and targeting to the cell surface are not disturbed by G12R and R32W. Subsequently, we assessed the functional properties of the mutant proteins by studying the transfer of 6-carboxy-fluorescein between cells. Cells expressing wildtype or R32W proteins established comparable levels of intercellular communication. This result provides experimental evidence that R32W is a nonconsequential polymorphism. In contrast, mutation G12R resulted in impaired intercellular coupling, suggesting that the mutant protein is not able to form functional gap junction channels. Further studies aim to determine whether this functional deficiency might be due to a disturbed oligomerization of hemichannels, or alternatively, due to changes of the functional properties of Cx31 channels.

## 773

**Mutations in the Connexin Gene *GJB4* (Cx30.3) Cause Erythrokeratoderma Variabilis (EKV)**

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EKV is an autosomal dominant genodermatosis characterized by persistent plaque-like or generalized hyperkeratosis and transient erythematous patches of variable size, shape and location. Recently, we demonstrated that EKV is caused by mutations in *GJB3* encoding the gap junction protein connexin-31 (Cx31) on 1p35.1. However, two-thirds of all families had no detectable mutations in *GJB3* or in the colocalized connexin genes *GJB5* and *GJA4* despite convincing evidence for locus homogeneity. We report here the identification of 4 distinct mutations in another connexin gene *GJB4* (Cx30.3) at this cluster in 5 EKV families. All heterozygous missense mutations completely cosegregated with the disease and altered highly conserved residues. One of them, G12D, lies in the N-terminus and may interfere with the gating polarity of Cx channels. Other mutations (T85P, F137L, F189Y) affected the transmembrane domains of Cx30.3, thus potentially hindering regulation of voltage gating and kinetics of channel closure. All *GJB4* mutations resulted in localized hyperkeratosis, while some corresponding mutations in *GJB3* were associated with severe, generalized skin involvement. Nevertheless, we observed considerable intrafamilial variability in 2 multiplex families, suggesting the influence of other genetic and environmental factors. Sequence analysis further revealed 3 missense substitutions and a common frameshift deletion within the *GJB4* coding region, which might represent either nonconsequential polymorphisms or recessive mutations, and as such, could contribute to the phenotype. Our results demonstrate genetic heterogeneity in EKV, and emphasize that intercellular communication mediated by Cx31 and Cx30.3 is crucial for epidermal differentiation.

## 774

**Erythrokeratoderma Variabilis is Heterogeneous – Mutation in the Novel Connexin 30.3 Gene**

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Erythrokeratoderma variabilis (EKV) is an autosomal dominant keratinization disorder first described by Mendes da Costa in 1925. It begins in early life and is characterised by migratory erythematous lesions which sooner or later turn into fixed keratotic plaques. So far, all EKV families have been mapped to chromosome 1p34-p35 and mutations in connexin 31 (Cx31) gene have been reported in some but not all families suggesting that another gene may be involved in EKV. We studied a large Israeli pedigree of Kurdish origin with annular EKV. After having mapped the disorder to chromosome 1p34-p35, we found no mutations in Cx31, Cx31.1 and Cx37 genes. Further investigation revealed a heterozygous T to C transition in the novel human Cx30.3 gene which localises on chromosome 1p34-p35. This nucleotide change cosegregated with the disease and was not found in 200 normal alleles. The resulting missense mutation (F137L) concerns a highly conserved phenylalanine in the third transmembrane region of the Cx30.3 molecule. This domain is known to be implicated in the wall formation of the gap junction pore. RT-PCR analysis revealed expression of human Cx30.3 primarily in keratinocytes *in vivo* and in culture, but also in fetal bladder and adult kidney. Our results show that mutations of the novel Cx30.3 gene are causally involved in EKV and point to genetic heterogeneity of this disorder. Furthermore, the disease in our pedigree may present a new type of annular EKV similar but not identical to genodermatose en cocardes of Degos.

## 775

**Papillon-Lefèvre Syndrome: Mutations and Polymorphisms in the Cathepsin C Gene**  
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The Papillon-Lefèvre syndrome (PLS), inherited in an autosomal recessive pattern, manifests with palmoplantar keratoderma and early, destructive periodontitis which may be evident during the first four years of life. Recently, mutations in the gene encoding cathepsin C (CTSC), a lysosomal cysteine protease, have been disclosed in a limited number of families with PLS. In this study, we have examined two consanguineous multiplex families with PLS, and evaluated the CTSC gene for mutations. Family 1 of Puerto Rican origin consisted of 4 affected and 7 unaffected siblings; the parents were clinically normal. Family 2 from Japan had 2 affected individuals; their parents were clinically unaffected. The mutation detection strategy consisted of PCR amplification of all 7 exons and the flanking intronic sequences of the CTSC gene, followed by direct nucleotide sequencing of the PCR products. This strategy identified missense mutations, W39S and G301S, in Families 1 and 2, respectively, affecting amino acid residues which were conserved between human, a number of vertebrate, and *Schistosoma japonicum* sequences within the cathepsin C polypeptide. The affected individuals were homozygotes, while heterozygous carriers (parents) of the mutations were clinically unaffected, confirming the recessive nature of the mutations. The W39S mutation resides within the N-terminal propeptide portion of the CTSC polypeptide which is cleaved off during post-translational processing. Thus, this mutation may interfere with the transport of the proform polypeptide from RER to lysosomes or impede the proteolytic processing of the polypeptide. The G301S mutation renders the enzyme catalytically inactive. In addition to these mutations, nonpathogenic polymorphisms were detected in the human gene. Addition of novel CTSC mutations into the expanding PLS mutation database allows further refinement of genotype/phenotype correlations towards understanding the molecular basis of this severe, syndromic genodermatosis.

## 777

**Candidate Genes for Alopecia Areata: Significant Association with HLA-Class II and Exclusion of CGRP, CD8, TNF- $\alpha$  and IL1-RN**

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Alopecia Areata (AA), patchy hair loss, may progress to total scalp or body hair loss, and affects 1–2% of the population. AA is a T cell mediated immune response directed at hair follicles, and may be another organ specific autoimmune disease, like diabetes, in which there is failure of self-tolerance. The study cohort consists of families identified through several Dermatology Clinics at Texas Medical Center, Houston, TX and through a web-site. Presently, a total of 118 multigeneration families with at least one affected member have been collected. We selected 5 candidate genes (CG) for this analysis: HLA Class II DR and DQB, TNF- $\alpha$  (tumor necrosis factor alpha), T-cell differentiation antigen CD8, CGRP (calcitonin gene-related peptide), and IL1-RN (interleukin 1 receptor antagonist). These CG are located on 6p21.3, 2p12, 11p15.1-p15.2, and 2q14.2, respectively. We used the transmission disequilibrium test (TDT) to test for association between alleles at specific candidate loci and risk of AA in 204 nuclear families. The results showed an association between the alleles of HLA DQB ( $p=0.00033$ ) and DR ( $p=0.00025$ ) but not for TNF- $\alpha$  ( $p=0.33$ ), CD8 ( $p=0.2$ ), CGRP ( $p=0.80$ ) and IL1-RN ( $p=0.48$ ). As with other organ-specific autoimmune diseases, the HLA locus appears to be a major determinant contributing to the pathogenesis of AA.

## 779

**Characterisation of the Upstream Regulatory Region of Type II Hair-Specific Keratin Genes**

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Several hair-specific keratin genes have been characterised and they exist, together with the other keratin genes, in two separate clusters on chromosomes 12 (type II) and 17 (Type I). The hair-keratin genes are specifically expressed in the growing hair shaft and nail plate. They are also modulated during the hair-growth cycle being down regulated during catagen, no expression during telogen and up regulated during early anagen. The elements that regulate both the tissue specific and temporal expression and unknown but must reside in the noncoding region up-stream, within or down-stream of the structural gene. In order to investigate the regulation of these genes, we have isolated PAC and cosmid clones, which make up a 120-kb contig of chromosome 12q13. This contains three complete functional type II hair-specific keratin genes (KRT-Hb1, KRT-Hb3 and KRT-Hb6) and we have sequenced the upstream regulatory regions of these genes. The PAC and cosmid clones were isolated by screening genomic libraries with a PCR generated 1 kb fragment of KRT-Hb3 and KRT-Hb1 genes. Clones were characterised by Southern blotting, long-range PCR, subcloning and sequencing. A 120-kb contig was assembled and subclones containing up to 4 kb of the regulatory regions of KRT-Hb1, KRT-Hb3 and KRT-Hb6 were made and sequenced. The sequences were analysed using the Transfac database (release 5.0) and prospective regulatory elements identified. Portions of the regulatory region (1 kb, 2 kb and 3 kb) of Hb3 and Hb6 were subcloned into a luciferase reporter gene vector (Promega pGL3) and transfected into Cos cells. The genes are about 10 kb apart and we have identified possible regulatory elements (AP1, CEBP, cRel, CHOP, AP2 and NF2B) up to 4 kb from the transcriptional start site. Preliminary reporter gene assays suggest some of these elements are functional in regulating the expression of these type II hair-specific keratin genes.

## 776

**Expression Analysis of SLURP-1, the Gene Responsible for Mal de Meleda**

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Mal de Meleda (MDM) is a rare autosomal recessive skin disorder characterised by transgressive palmoplantar keratoderma and keratotic skin lesions. We report here the expression analysis of the secreted Ly-6/uPAR related protein 1 (= SLURP-1), mutations of which are causing MDM. SLURP-1 is a member of the Ly-6/uPAR protein superfamily of which most members have been localised to a cluster on chromosome 8q24.3. The amino acid composition of SLURP-1 is homologous to that of cytotoxins such as snake venom neurotoxins and cardiotoxins. Members of the Ly-6/uPAR superfamily have been implicated in transmembrane signal transduction, cell activation and cell adhesion. Expression of SLURP-1 transcripts was investigated in cultured keratinocytes and tissue samples from normal and affected individuals. Northern blot analysis showed strong expression of SLURP-1 mRNA in cultured keratinocytes derived from normal control plantar skin but none in cultured keratinocytes established from involved plantar skin of patients. No transcripts were detected in cultured keratinocytes from normal trunk skin or in biopsies from control trunk epidermis. Using a more sensitive technique, RT-PCR analysis detected SLURP-1 mRNA in cultured keratinocytes established from skin biopsies of different body sites in both controls and MDM patients. In biopsies from normal subjects, SLURP-1 mRNA was found to be expressed in epidermis, foreskin, scalp skin and fetal bladder, but not in the other tissues tested. Interestingly, in one MDM patient with a homozygous splice site mutation we found two RT-PCR products of different sizes compared to controls indicating that no normal SLURP-1 mRNA is formed in this patient. These results indicate that SLURP-1 is mainly expressed in plantar, and most likely, in palmar skin which corresponds to the predominant sites of MDM lesions.

## 778

**A Nonsense Mutation in MSX1 Causes Tooth and Nail (Witkop) Syndrome**

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Tooth and Nail Syndrome (TNS) is a rare autosomal dominant disorder. It can be distinguished from other types of ectodermal dysplasias (EDs) in that individuals with TNS have congenitally missing permanent and/or primary teeth and nail dysplasia, but sweat glands and hair are normal. To identify a mutation that is responsible for TNS, we used candidate gene linkage analysis and sequencing in a three-generation family affected by the disorder. We considered the transcription factor MSX1 an excellent candidate gene because Mx1 is highly expressed in developing murine tooth buds and forming nail beds of mouse digits. Furthermore, tooth development is arrested in Mx1 knockout mice, and mutations in MSX1 have been shown to be associated with congenitally missing teeth in two unrelated families. We established strong linkage between the TNS phenotype and polymorphic microsatellite markers surrounding the MSX1 gene, and we sequenced exons 1 and 2. We found a C-A transversion at nucleotide position 2143 in exon 2. This results in an S202X nonsense mutation and creates an NheI restriction site. Using restriction fragment length analysis, we found that the mutation is present in all affected members, but not in unaffected members, and 132 normal control chromosomes. This nonsense mutation is located in the homeodomain of MSX1 and is predicted to cause a truncated protein that cannot bind target DNA. We hypothesize that haploinsufficiency is the most likely mechanism by which the mutation causes TNS. In addition, we have found that Mx1 knockout mouse embryos have a delayed development of proximal nail fold, and nail plates in Mx1 knockout mice are much shorter and thinner than that of their wild-type littermates. The striking resemblance between tooth and nail phenotype in our family and that of Mx1 knockout mice strongly supports the conclusions that S202X MSX1 causes Tooth and Nail Syndrome and Mx1 is necessary for normal tooth and nail development.

## 780

**Evidence for Extensive Locus Heterogeneity in Naxos Disease**

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Naxos disease is a rare inherited defect of the hair shaft resulting in sparse and dystrophic hair. The clinical picture is associated with a triad of woolly hair, thickened palms and soles (keratoderma) and heart involvement. The hair involvement is unique, and is characterized by woolly, curly, rough and light colored scalp hair and sparse eyebrows. Affected individuals suffer at young age from severe psychological and social disability and later also develop a severe heart disease that may end with arrhythmia and premature sudden death. This autosomal recessive associated triad was originally described in patients from the Greek island of Naxos by Protonotarios *et al* in 1986 and by Barker *et al* in 1998. In 1998, Luis Carvajal-Huerta described 18 patients from Ecuador with a similar autosomal recessive triad, combined of epidermolytic palmoplantar keratoderma with woolly hair, dilated cardiomyopathy and detailed the skin manifestations. Recently, the gene for the Greek families was mapped to 17q21, and mutations in two different genes, plakoglobin and desmoplakin, have been identified as responsible for the disease in the Greek and Ecuadorian families, respectively. We have identified two additional large consanguineous families from Israel and obtained DNA from most members of these families. First, we excluded a mutation in the desmoplakin and plakoglobin genes by sequence analysis, suggesting that at least one additional gene underlies Naxos disease in these families. We then performed cosegregation studies using microsatellite markers covering the keratin clusters on chromosomes 12 and 17, the desmoyokin locus (11q13.1), and the desmocollin/desmoglein cluster (18q12.1). We excluded these as candidate genes, since none of these regions showed cosegregation with the disease trait using homozygosity mapping in these inbred families. We have now initiated a whole genome search to identify the gene(s) causing Naxos disease in our families.

## 781

### Proliferation Status of Nevi in Utah Kindred Members with and without CDKN2A Mutations

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Approximately 40% of familial melanoma is caused by a mutation in cyclin dependent kinase inhibitor 2 A (CDKN2A, p16), a tumor suppressor that acts by regulating the cell cycle via the retinoblastoma (Rb) tumor suppressor pathway. One of several melanoma-prone families studied more than 10 years ago at the University of Utah was instrumental in establishing the melanoma susceptibility locus on chromosome 9p21 and later confirming the relevance of the mutation in CDKN2A. This family carries a coding mutation of CDKN2A, V126D, that cosegregates with disease. The mutated protein product is temperature sensitive, showing approximately 33% of normal CDK4 binding at 30 degrees and dropping to less than 10% at 42 degrees. Defective binding to CDK4 results in hyperphosphorylation of Rb, and E2F-mediated re-entry into the cell cycle. DNA topoisomerases are enzymes that control DNA topology. Topoisomerase II- $\alpha$  (topo II) is required for DNA replication, is cell cycle specific, and is a well-established marker of cellular proliferation. We hypothesized that family members carrying the CDKN2A mutation would have nevi in a higher proliferative state than family members that did not harbor the mutation. One hundred four nevi from 54 kindred members were immunostained for topo II. The slides were evaluated in blinded fashion by two pathologists who performed topo II index counts on each nevus. Results were evaluated in conjunction with the kindred members' mutation status. Preliminary results suggest that the cellular proliferation index of nevi as determined by topo II immunostaining cannot be used to predict CDKN2A mutation status. Future studies with a more sensitive marker of proliferation are warranted.

## 783

### A New Locus for Hermansky-Pudlak Syndrome in Puerto Rico

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Hermansky-Pudlak Syndrome (HPS) is an autosomal recessive disorder characterized by oculocutaneous albinism, a bleeding diathesis due to platelet storage pool deficiency, and lysosomal accumulation of ceroid lipofuscin. HPS occurs with a high frequency in north-west Puerto Rico (PR), where 1 in 21 individuals is a carrier. HPS patients from north-west PR are homozygous for a 16-bp duplication in exon 15 of the HPS1 gene; this mutation is associated with significant risk of pulmonary fibrosis. The objective of this study is to determine the phenotype and genotype of HPS patients from several part of the island and map the gene for a new subtype of HPS. Sixty-one of 76 patients displayed the 16-pb duplication in the HPS1 gene; all from North-west Puerto Rico. However, we found 15 patients from 8 families, all from central Puerto Rico, who lacked the 16-bp duplication or any other mutation on HPS1 as determined by cDNA sequencing. All patients studied lacked platelet dense bodies on electron microscopy, confirming the diagnosis of HPS. The patients from central Puerto Rico exhibited only mild visual defects and mild pigment dilution of hair, eyes and skin, with no pulmonary fibrosis. These findings differed significantly from HPS individuals with the 16-bp duplication in HPS1. Using homozygosity mapping on pooled DNA from 8 families from central PR, we mapped the putative new HPS susceptibility gene to a region of less than 2-cM in an area of the human genome homologous to a murine locus for a model of HPS. These results indicate the chromosomal location for a new HPS-causing gene and allow for physical mapping, gene identification, and mutation analysis of human HPS patients with currently unknown molecular defects.

## 785

### Altered Asian Skin Blanching Responses to Dermatologic Corticosteroids Containing Alcohol is Associated with an Alcohol Dehydrogenase 2 Polymorphism

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Previous research in our laboratory demonstrated that Asians required 3–10X the dose as Caucasians to produce the same maximal skin blanching response (SBR) with dermatologic corticosteroid (DC) products containing alcohols. The ethnic SBR differences were independent of DC uptake into skin. A possible genetic basis for the SBR differences was investigated with three candidate genes that encode for (1) 11b hydroxy steroid dehydrogenase 1 (11bHSD-1) and 2 (11bHSD-2): enzymes involved in corticosteroid metabolism that regulate the amount of DC available to the corticosteroid receptor and (2) alcohol dehydrogenase 2 (ADH-2): an enzyme responsible for alcohol metabolism and associated with the flushing response. The sequences of these genes were determined from extracted peripheral blood DNA in 9 Caucasian and 7 Asian subjects previously evaluated for SBR to seven 0.05% betamethasone dipropionate products with and without alcohol(s) in their vehicles. Comparisons of the DNA sequences revealed no differences in exons 1–5 of 11bHSD-1 and no differences in exons 2–5 of 11bHSD-2 between the two ethnic groups. However, the documented arginine to histidine polymorphism of amino acid 47 in ADH-2 was observed in 100% of the Asian subjects evaluated. These data suggest the differential SBR to DC between Asians and Caucasians may reflect the effect of metabolism of alcohols contained in the products rather than differences in DC metabolism itself.

## 782

### Loss of Heterozygosity at 1p36 and 9p22–21 Regions in the Human Melanocytic Skin Lesions

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Analysis of loss of heterozygosity (LOH) in tumors is a powerful tool for mapping the sites of tumor suppressor genes (TSGs). We used PCR-based microsatellite assay to analyze 30 benign melanocytic nevi (BN), 60 melanocytic dysplastic nevi (MDN), and 22 vertical growth phase melanomas (CMM) for the presence of LOH at 1p and 9p regions. LOH was found at 1p and 9p in CMM and MDN but not in BN. At 1p36 region, LOH was found in 8/22 (36%) CMM and 9/60 (15%) MDN. 1p36 deletion mapping defined 2 deletion regions centered at D1S214 and D1S228 loci. At 9p22–21 regions, LOH was detected in 7/22 (32%) CMM and 9/60 (15%) MDN. The 9p deletion pattern revealed LOH hot spots at D9S171 and D9S169. Our study suggests the inactivation of TSGs at 1p and 9p regions during melanoma tumorigenesis.

## 784

### CpG Island-Specific Hypermethylation in Peripheral Blood Lymphocytes of Systemic Lupus Erythematosus

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DNA methylation, especially methylation in CpG island of genome, is one of the most important mechanisms in epigenetic regulation of gene expression. We developed highly sensitive method of evaluating CpG island-specific methylation of whole genome, nonisotopic cytosine extension assay (NICEA), and reported previously. We analyzed CpG island methylation status of peripheral blood lymphocytes of systemic lupus erythematosus (SLE) and compared with cutaneous LE (discoid LE and LE profundus). Genomic DNA of peripheral blood lymphocytes of SLE (n = 15), CLE (n = 10), and control (n = 10) was extracted. The genomic DNA was digested with BssHII, HpaII, or MspI, and subjected for NICEA study. BssHII/MspI, represents nonmethylated portion of CpG island vs. whole CpG, and BssHII/HpaII, represents nonmethylated portion of CpG island vs. nonmethylated portion of whole CpG, were calculated. BssHII/MspI was decreased significantly, compared with CLE; BssHII/HpaII was decreased significantly, compared with the control. The difference between CLE and the control was not significant, statistically. In conclusion, CpG island of peripheral blood lymphocyte was hypermethylated in SLE, but not in CLE. The hypermethylation of CpG island can be a mechanism of dysregulation in lymphocyte function of SLE.

## 786

### Gene Expression of MMP-1 and MMP-13 is Up-Regulated with Worsening Venous Reflux in Patients with Venous Insufficiency

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Venous insufficiency is a spectrum of disease that varies from varicose veins to chronic venous ulceration. Worsening venous reflux and elevated ambulatory venous pressure are characteristic of this state. We were interested in investigating if elevations in venous pressure correlated with changes at the molecular level in the vein walls of these patients. One centimeter segments of greater saphenous vein (GSV) were obtained from patients undergoing ligation and stripping for venous insufficiency (n = 15). All patients had incompetence of the GSV by color flow duplex and elevated venous filling index (VFI) by air plethysmography. Vein specimens were analyzed for MMP-1, 3, 13, tryptase and GADPH mRNA using RT-PCR. Quantification of MMP-1, 13 (active/latent forms) and tryptase was performed using Western blot analysis. Gelatin zymography was performed looking for MMP-1 or MMP-13 activity. Western blots were analyzed using scanning densitometry and values expressed as the median densitometric index. Spearman's rank correlation was used for statistical analysis. MMP-1, MMP-13 and tryptase mRNA was expressed in all segments of GSV. MMP-3 mRNA however, was not found in any segment. We found a direct correlation between elevated MMP-1 (latent form) gene expression and worsening venous reflux ( $r_s = 0.547$ ) ( $p = 0.043$ ). Similarly we found a significant difference in the quantity of MMP-13 protein (active form) ( $r_s = 0.618$ ) ( $p = 0.019$ ) and MMP-13 protein (latent form) ( $r_s = 0.618$ ) ( $p = 0.027$ ) and elevated VFI. We found no such correlation however, in the quantity of tryptase in segments of GSV. This study shows a direct correlation exists between worsening venous reflux and the up regulation of the matrix remodeling proteins MMP-1 and MMP-13. We believe this is the first example connecting physiologic observations with induced molecular changes, ultimately leading to venous pathology.



## 787

**Birt-Hogg-Dubé Syndrome has an Increase Risk for Renal Neoplasms and Spontaneous Pneumothorax**B. Zbar, J. Toro, M. Turner, G. Glenn, P. Duray, P. Choyke, and M. Linehan  
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The Birt-Hogg-Dubé syndrome, a genodermatosis characterized by hamartomas of the hair follicle, has been associated with renal and colonic neoplasms, and spontaneous pneumothorax, but the risk of developing these disorders is unknown. To determine risk, we recruited families on the basis of a diagnosis of the Birt-Hogg-Dubé syndrome and compared the frequency of renal and colonic tumors, and of spontaneous pneumothorax in patients affected with the Birt-Hogg-Dubé syndrome, to the frequency of these manifestations in their unaffected siblings (controls). To determine the number of individuals affected with renal or colon tumors, we added the number of individuals with a history of renal or colon neoplasia to the number of individuals found to have tumors by screening procedures. We studied 222 subjects from 26 families affected with the Birt-Hogg-Dubé syndrome; there were 110 affected and 112 unaffected individuals. 15/110 (14%) patients affected with the Birt-Hogg-Dubé syndrome had renal tumors. In comparison, 2/112 (2%) control subjects had renal tumors ( $p = 0.0009$ ). The risk of developing renal tumors in patients affected with the Birt-Hogg-Dubé syndrome as measured by the odds ratio was: 8.7 (95% confidence limits: 1.9–39.0). Renal tumors in patients with the Birt-Hogg-Dubé syndrome were predominantly chromophobe or clear cell renal carcinomas. 25 of 110 (23%) patients with the Birt-Hogg-Dubé syndrome gave a history of spontaneous pneumothorax; 0/112 (0%) of unaffected members of Birt-Hogg-Dubé families gave a history of spontaneous pneumothorax. The risk of developing spontaneous pneumothorax in patients affected with the Birt-Hogg-Dubé syndrome was: 67.1 (95% confidence limits: 4.0–1118). Pulmonary cysts were detected by high resolution thoracic CT scans in 83% of patients affected with the Birt-Hogg-Dubé syndrome, and in 10% of controls. There was no significant difference in the frequency of colon polyps (22% vs. 19%,  $p = 0.75$ ) or colon carcinomas in Birt-Hogg-Dubé affected compared to non affected individuals. Birt-Hogg-Dubé syndrome confers an increased risk for the development of renal tumors, lung cyst and spontaneous pneumothorax. We found no increase in risk for colon polyps or colon carcinomas.

## 789

**Identification of a Novel D132H Missense Mutation in the Flavin-containing Monooxygenase 3 Gene in Trimethylaminuria**S. Kobayashi, J. Kim,\* H. Lawley,\* S. Tjota,† P. Fennessey,† G. Preti, and S. Fakhrazadeh  
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Trimethylaminuria (TMAU) is a metabolic disorder that results from deficiency of the flavin-containing monooxygenase 3 (FMO3) enzyme. This leads to an inability to metabolize trimethylamine (TMA), which accumulates and may sporadically impart a fishy or foul smell. Affected individuals frequently present to dermatologists complaining of skin malodor. An autosomal recessive inheritance pattern for this disorder has been observed and mutations within the FMO3 gene that reduce or ablate its product's function have been identified. However, milder forms of the disease that result from mutation of one FMO3 allele or even heterozygosity for certain polymorphisms that may reduce FMO3 activity have been described. Also, factors such as diet, gut flora content, and time in the menstrual cycle may influence the expression of milder forms. We have performed mutation screenings on subjects that presented with malodor and displayed reduced capacity to generate trimethylamineoxide (TMAO) after choline challenge. Each coding exon of the FMO3 gene (exons 2–9) was amplified from genomic DNA and subjected to direct sequencing. We have identified a female African-American proband who displays 47% of normal FMO3 activity and carries a novel D132H alteration in exon 4. We detected only the "g" to "c" change responsible for this amino acid substitution at nucleotide 73 of exon 4 and we observed no variation at polymorphic sites elsewhere in the FMO3 gene. This suggested that either she may carry two identical FMO3 alleles or she may have a deletion of one FMO3 allele. Southern blot analysis revealed no evidence for allele deletion compared to controls. Taken together, these observations indicate that the D132H mutation likely accounts for reduced FMO3 activity in this individual. The nucleotide substitution introduces a new NLAIII restriction site into exon 4 coding sequences. Forty control subjects, of which 16 are African-American, lack this NLAIII site, indicating that this alteration unlikely represents a polymorphism. In sum, we have identified a novel D132H missense mutation in the FMO3 gene in an individual with TMAU. To our knowledge, this represents the first report of mutation in FMO3 associated with TMAU in an African-American individual.

## 791

**Induction of Apoptosis by Telomere 3' Overhang Specific Oligonucleotides in Human Melanoma Cells**N. Puri, M. Eller, H. Byers, and B. Gilchrist  
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The 3' end of each telomere is a single-stranded overhang of repeats of TTAGGG and is proposed to form a loop structure at the chromosome ends. Considerable evidence suggests that exposure of the telomere overhang by experimental disruption, DNA damage, progressive telomere shortening or provision of homologous DNA fragments is a signal for DNA damage response such as apoptosis. To explore the mechanism and possible therapeutic utility of these responses, we designed 11-base oligonucleotides homologous, complementary and unrelated to the telomere overhang (11mer-1, -2, -3, respectively) and added them separately to cultures of 5 human melanoma cell lines. By FACS, 3 of these cell lines showed an increase in apoptotic cells after treatment with 11mer-1, compared to diluent, 11mer-2 or -3. In the most responsive cell line, after 72 h of treatment with 11mer-1, up to 32% of the cells were apoptotic, compared to 2% of the diluent- or 11mer-2- or 11mer-3-treated cells ( $p = 0.01$ ) and by 96 h, up to 64% of cells were apoptotic, compared to 5% of the controls ( $p = 0.01$ ). Apoptosis was also detected by TUNEL assay in 11mer-1-treated melanoma cells. In contrast, the 11mer-1 did not induce apoptosis in normal human melanocytes. To study the mechanism of apoptosis, a responsive line (MM-AN) and a resistant line (r.p.m.-EP) were treated with the oligonucleotides, proteins were collected and analyzed by Western blot. An increase in the E2F1 transcription factor was seen after 24 h of treatment with 11mer-1 but not -2 or -3 in the MM-AN cells, followed by an increase in the level of the p53-related protein p73, known to cooperate with E2F1 in apoptosis. No change in p53 level was detected. No induction of E2F1 or p73 was detected in r.p.m.-EP cells. These data show that DNA oligonucleotides homologous to the telomere overhang induce apoptosis in a subset of melanoma cells, likely by the induction of E2F1 and p73, but not in normal melanocytes and also suggest that these DNAs may have therapeutic potential.

## 788

**Mutations in p63 and Genotype-Phenotype Correlation in AEC and EEC Syndromes**J. McGrath and H. van Bokhoven\*  
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Linkage and mutation analyses have identified heterozygous missense mutations in the DNA-binding domain of the p63 gene as the major cause of EEC (ectrodactyly, ectodermal dysplasia, clefting) syndrome. Other autosomal dominant ectodermal dysplasia syndromes have also mapped to the p63 locus at 3q27 suggesting allelic heterogeneity. In this study, we assessed whether AEC (ankyloblepharon, ectodermal dysplasia, clefting) syndrome also results from mutations in p63. Genomic DNA from 2 patients with EEC syndrome and 8 patients with AEC syndrome was analyzed by PCR amplification of the 15 exons of p63 using primers sited in flanking introns. PCR products were sequenced directly and sequence variants were verified by restriction digestion and exclusion in 100 control chromosomes. In the EEC patients, we identified heterozygous R279H and R304W substitutions in exons 7 and 8, respectively. In the AEC patients we detected the heterozygous mutations L518V, L518F, C526G, C526W, G534V, T537P, Q540L and I541T, all in exon 13. Eight of the 10 p63 mutations occurred de novo. The amino acid changes in the EEC patients are located in the core DNA-binding domain of p63 and have been recorded in two other unrelated patients. Indeed, these two residues correspond to the amino acids in p53 (R248 and R273) that are most frequently mutated in human tumors. The amino acid substitutions in the AEC patients establish the molecular basis of this genodermatosis, and confirm allelic heterogeneity for p63 mutations in EEC and AEC syndromes. All AEC mutations occurred within the SAM (steric-alpha-motif) domain, part of p63 which is important in protein-protein interactions, but for which an equivalent domain in p53 is lacking. In summary, these data provide evidence for a specific genotype-phenotype correlation in this group of inherited ectodermal dysplasia syndromes.

## 790

**Mechanism of S-Phase Arrest by Telomere 3' Overhang Specific DNA**M. Eller, N. Puri, G. Li, and B. Gilchrist  
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Telomeres, repeats of TTAGGG at chromosome ends, are proposed to act as a biological clock and cancer prevention mechanism. The telomere 3' end is a single-stranded overhang, normally concealed in a loop structure. Telomere disruption triggers apoptosis and other DNA damage responses, as does treatment of cells with DNA oligonucleotides that share homology with the telomere overhang. In normal cells, the S-phase arrest induced by ionizing radiation (IR) requires phosphorylation of p95/Nbs1, the protein mutated in Nijmegen Breakage Syndrome. We therefore examined the role of p95/Nbs1 in the telomere overhang oligonucleotide-induced S-phase arrest. A human T cell line (Jurkat), a squamous cell carcinoma line (SCC12F), an osteosarcoma cell line (Saos-2), normal neonatal human fibroblasts, NBS fibroblasts and age-matched control fibroblasts were treated with 11-base oligonucleotides either homologous, complementary, or unrelated to the telomere overhang (11mer-1, -2, -3, respectively). After 48 h, cells were collected for FACS analysis. Cells treated with 11mer-2 or -3 had similar profiles compared to diluent-treated controls. However, cells receiving 11mer-1 showed increases of 100% (Jurkat), 355% (neonatal fibroblasts), 120% (SCC12F) and 68% (Saos-2) in S-phase cells ( $p < 0.01$ ). NBS cells showed only a 23% ( $p < 0.07$ ) increase while the control fibroblasts increased by 85% ( $p < 0.01$ ). As previously shown for IR, p95/Nbs1 in cells treated with the 11mer-1 demonstrated a shift to a higher molecular weight concomitant with the S-phase arrest and this shift was eliminated by treatment with a serine/threonine phosphatase, implicating phosphorylation of p95/Nbs1. Because Jurkat cells have a nonfunctional p53 pathway and Saos-2 cells are p53-null, the S-phase arrest induced by 11mer-1 is independent of p53. These data suggest that p95/Nbs1 mediates inhibition of DNA replication induced by telomere 3' overhang DNA and support the hypothesis that exposure of this sequence is a physiologic signal for DNA damage responses.

## 792

**Induction of Replicative Senescence Markers by Telomere 3' Overhang Specific DNA**G. Li, M. Eller, and B. Gilchrist  
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Mammalian cells have a tightly regulated program of replicative senescence, suggested to be a fundamental defense against cancer and controlled largely by telomere length. Telomeres, tandem repeats of TTAGGG, cap the ends of chromosomes and shorten with cell division. Disruption of the telomere loop structure exposes the telomere 3' overhang and mimics cellular responses to DNA damage. Treatment of cells with single-stranded oligonucleotides homologous to the telomere 3' overhang also induces DNA damage-like responses. To determine if replicative senescence is among these responses, normal human fibroblasts were treated with 11-base oligonucleotides either homologous to or complementary to the telomere 3' overhang or with diluent alone and were stained for senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -Gal) activity. Cells treated with the telomere 3' overhang homolog showed 6-fold more SA- $\beta$ -Gal positive cells compared to cells treated with diluent or the complementary sequence ( $61 \pm 10\%$ ,  $7 \pm 2\%$  and  $10 \pm 2\%$ , respectively;  $p = 0.01$ ). Homolog-treated cells also manifested a senescent morphology, with many large spread cells. To look for other markers of senescence, paired fibroblast cultures were treated for 3 days as described above or serum-starved to induce quiescence and then serum-stimulated for 24 h, collected and analyzed by Western blot. Cells treated with the overhang homolog, compared to diluent- or complement-treated cells or to previously quiescent cells, overexpressed p53 and the senescent cell-derived inhibitor of pRb phosphorylation, p21 (SDI-1) and failed to phosphorylate pRb in response to serum stimulation. Furthermore, these cells expressed higher levels of the promyelocytic leukemia protein (PML) known to be up-regulated during ras-induced senescence of these cells. The data strongly suggest that telomere shortening during aging induces senescence by destabilizing the telomere loop and hence exposing the 3' overhang and that this physiologic signal can be mimicked by homologous DNA fragments.

## 793

**p33ING1 Role in DNA Repair**

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ING1 is a new tumor suppressor that has no significant homology with other genes. However, it shares many biological functions with those of p53. Some of its functions include G1-phase growth arrest and apoptosis in cooperation with p53, replicative senescence, chemosensitivity, and anchorage-dependent growth. We have recently shown that the regulation of ING1 expression is not dependent on p53 *in vivo* and that ING1 expression is induced by UV irradiation in a dose-/time-dependent and tissue-specific manner. To investigate if ING1 plays a role in DNA repair, we performed the CAT assay where a UV-damaged plasmid containing the chloramphenicol acetyltransferase reporter gene (pCMV-cat) was cotransfected with either vector, ING1, or antisense ING1 expression vector into mammalian cells. The activity levels of the reporter gene were then used as an indicator of the extent of repair. Our data demonstrates that cells transfected with the ING1 gene have 2–3 fold increase in the rate of repair of the UV-damaged plasmid compared to the vector and antisense controls. In addition, results from the radioimmunoassay show that global genomic DNA repair is enhanced by approximately 50% in ING1-transfected cells compared to the vector-transfected control cells. It is interesting to note that this repair capability of ING1 is, to a large extent, dependent on the status of p53, as enhancement in ING1-mediated repair is reduced significantly when p53 is knocked out by transfection with a dominant-negative p53 construct. Finally, results from immunoprecipitation suggest that there is a weak physical association between ING1 and GADD45, a DNA-damage-inducible gene involved in excision repair. These results strongly indicate that ING1 participates in nucleotide excision repair of UV-damaged DNA.

## 795

**Transcriptional Fine-Tuning: The Nuclear Receptor's Hierarchy in the Regulation of Keratin Gene Expression**

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We have shown previously that the glucocorticoids (GCs), thyroid hormone (T3) and retinoic acid (RA) suppress the expression of the disease-associated keratin genes (K5, K14, K6, K16 and K17) through a unique molecular mechanism that involves particular nuclear receptor configurations and keratin response elements (KREs). One of the remaining questions is how these three hormone receptors maintain such transcriptional regulation in their natural environment, i.e. when they are all simultaneously present? To determine how the simultaneous presence of multiple receptors changes the binding pattern to keratin REs and regulation of keratin gene expression, we have performed footprinting, gel-shift and cotransfection experiments with the combinations of T3R, GR and RAR. We have found that T3R and GR can simultaneously bind the KRE using gel-shift and footprinting experiments. Furthermore, when both T3R and dexamethasone (DEX) are simultaneously present T3R blocks keratin gene regulation by DEX. Conversely, DEX does not affect the induction of keratin gene expression mediated by unliganded T3R. We conclude that T3R dominates over GR in regulating keratin gene transcription. Furthermore, RAR blocks the regulation by T3R when both are simultaneously present. We conclude that there is the hierarchy in receptor regulation of keratin genes in which RAR dominates over T3R and T3R dominates over GR. The role of the KREs is to allow interplay of multiple nuclear receptors, thus creating a gradient of hormonal action. Such receptor interactions provide fine-tuning of transcriptional regulation of keratin genes. The sequences of KREs dictate the hierarchy of nuclear receptors responding to a large variety of physiological conditions under which the suppression of these genes is required. This hierarchy is extremely important in epidermis, a target tissue that is exposed to and depends on regulation by multiple hormones.

## 797

**Transcriptional Regulation of Mouse Col7a1 Gene in Keratinocytes**

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The transcriptional control of the mouse Col7a1 gene that codes for the constitutive subunit polypeptide of type VII collagen, 1 pro  $\alpha 1$  (VII), was studied in a mouse keratinocyte cell line. This keratinocyte cell line that biosynthetically resembles basal keratinocytes, was established by spontaneous immortalization of a primary keratinocyte cell culture from a 129Sv mouse epidermis. Analysis of Col7a1 promoter/CAT constructs in this cell line revealed the presence of a region between -340 and -560 that was required for high level expression in this cell system. There was also a region between -560 and -1590 that possessed down-regulatory elements. Mutagenesis identified regions in the proximal promoter between the transcription start site and -130, and distal promoter between -340 and -560 that are required for high levels of promoter activity. Electrophoretic mobility shift assays determined that factors similar to Sp1, Sp3, AP1, and AP2 bind to discrete *cis*-elements in these regions. Such factors have been implicated in the expression of other epidermal genes, as well. It has been shown by others (Gardella R *et al*, *Hum Mut* #364, 2000) that mutation of a single Sp1 site at position -96 was associated with development of dystrophic EB. We show that a similar mutation in the mouse Col7a1 promoter results in decreased promoter function, demonstrating the requirement of Sp1 for basal Col7a1 gene regulation.

## 794

**The Role of Nucleotide Excision Repair Genes XPA and XPC in Melanoma Pathogenesis**

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Host response to ultraviolet (UV) exposures is a critical factor in cutaneous melanoma pathogenesis and defects in DNA repair pathways are associated with skin cancer development. Xeroderma pigmentosum (XP) is a genetic, skin cancer-prone, DNA repair-deficient disorder resulting from the inherited loss of function of one of seven XP nucleotide excision repair (NER) genes. A limited number of studies have suggested that the development of melanoma in XP is most frequently associated with XPA and XPC gene defects. The goal of this study was to explore the role of XPA and XPC in sporadic melanoma by screening for the frequency of loss of heterozygosity (LOH) detected at XPA (9q22.3) and XPC (3p25.1) gene loci. DNA from microdissected vertical growth phase, tumorigenic cutaneous melanomas (n=6) was PCR amplified with primers for microsatellite markers D9S287 (within 1.5 cM of XPA), and with D3S1597, D3S1263, D3S1585, D3S3726, D3S1293 (spanning a 12.8-cM region around XPC) and compared to DNA from normal tissue within the same specimens. PCR products were analyzed by capillary electrophoresis (ABI system 3100). A LOH index was calculated (peak height tumor allele 1/peak height tumor allele 2  $\div$  peak height normal allele 1/peak height normal allele 2) and LOH was defined by an LOH index of 1.5 or <0.5, indicating a 50% loss in the relative peak height of allele 2 or allele 1, respectively. For XPA, 3 of 6 cases were informative for D9S287, and 1 of the 3 informative cases demonstrated LOH at this marker. For XPC, 2 of 6 cases demonstrated LOH involving a 12.8-cM region flanking the XPC gene. The case demonstrating LOH for XPA also demonstrated LOH for XPC. Our preliminary results demonstrate that LOH involving XPA and XPC gene loci may be detected within a subset of cutaneous melanomas. This suggests that an acquired loss of function of XPA and/or XPC genes, resulting in DNA repair defects may contribute to sporadic melanoma development and progression. Further studies to confirm the mutation status of XPA, XPC and other XP NER genes are required to confirm and delineate the role of XP NER genes in the pathogenesis of cutaneous melanoma.

## 796

**An AP2 Site in the Mouse Trichohyalin Gene Promoter May Confer Tissue Specific Expression**

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Trichohyalin (THH) is a major structural protein in the inner root sheath of hair follicle, and also is involved in late stage of differentiation in the epidermis. To elucidate factors controlling its transcription, a series of CAT constructs containing several different size of inserts between -478 bp upstream from the transcription start site and the first exon (+36) of mouse THH (mTHH) gene were transfected into mouse hair follicle cells as well as epidermal keratinocytes. The results showed that as few as 250 bp are sufficient to confer strong promoter activity. Within this region, we found eight elements including three Sp1-like motifs (-234, -135 & -104), an AP1 site (-115), an AP2 site (-69), three unidentified motifs named E1 (-191), E2 (-160), and R1 (-126). Supershift assays revealed that Sp1, Sp3, c-Jun, AP2- $\alpha$ , AP2- $\gamma$  antibodies each bind to its corresponding motif. Separate mutations on E2 (-160), Sp1 (-135) and AP1 in the context of the pCAT (-250/+36) construct reduced CAT activity to basal level in both hair follicle cells and keratinocytes indicating these three elements are essential for promoter activity. E1 (-191) and the other Sp1 motifs (-234 & -104) are necessary but not sufficient to confer mTHH promoter activity. Furthermore, mutation of either R1 (-126) or AP2 showed 40% and 60% CAT activity increase over the wild type construct, respectively, in cultured hair follicle cells. However, mutation of AP-2 decreased CAT activity to basal level in keratinocytes implicating that AP-2 is a positive element. Therefore, AP2 site is necessary for mTHH expression in keratinocytes in addition to the E2 (-160), Sp1 (-135) and AP1 motifs. Thus, it is suggested that AP2 play opposite roles in mTHH transcription regulation in cultured hair follicle cells vs. keratinocytes. We suggest it may be involved in THH cell-type specific expression.

## 798

**Thapsigargin Suppresses Differentiation-Dependent Human Involucrin Promoter Activity by Reducing CCAAT/Enhancer Binding Protein-A DNA Binding**

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Human involucrin (hINV) is a keratinocyte differentiation marker expressed in the suprabasal epidermal layers. In cultured keratinocytes, hINV expression is increased by phorbol ester, an agent that promotes keratinocyte differentiation. Previous studies show that thapsigargin (TGN), an agent that depletes intracellular calcium stores, inhibits keratinocyte differentiation. We show that TGN inhibits the phorbol ester-dependent increase in hINV mRNA level and promoter activity. Inhibition is half-maximal at 10 nM and maximally at 100 nM, but basal hINV promoter activity is not regulated. Mutation of a functionally important CAATT-enhancer-binding protein (C/EBP) site within the hINV promoter proximal regulatory region eliminates the regulation, suggesting that TGN may effect C/EBP-dependent promoter activation. Consistent with this suggestion, TGN inhibits C/EBP $\alpha$ -dependent promoter activation via a mechanism that involves inhibition of C/EBP $\alpha$  binding to DNA. This reduction is observed without an accompanying change in C/EBP $\alpha$  protein level. As C/EBP $\alpha$  activity is absolutely required for optimal hINV promoter activity and gene expression (Agarwal *et al*, *JBC* 274:6190, 1999), this result suggests that TGN interferes with hINV expression by interfering with C/EBP transcription-factor function.

## 799

### Green Tea Polyphenol Increases Ap1 Factor-Dependent Human Involucrin (hINV) Gene Expression in Normal Human Keratinocytes

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(−)-Epigallocatechin-3-gallate (EGCG) is an important bioactive constituent of green tea that efficiently reduces epidermal cancer cell proliferation. This inhibition is associated with a reduction in activator protein 1 (AP 1) transcription factor level and activity. However, the effect of this candidate therapeutic agent on normal keratinocyte function has not been extensively explored. Our present studies suggest that EGCG is a potent regulator of normal keratinocyte function. To understand the mechanism of action, we studied the effects of EGCG on AP1 factor activity, and expression of the AP1-factor regulated human involucrin (hINV) gene. hINV is an established, well-studied marker of human epidermal keratinocyte terminal differentiation (Welter *et al*, *JBC* 270:12614, 1995; Crish *et al*, *JBC* 273:30460, 1998; Efimova *et al*, *JBC* 275:1601, 2000). hINV promoter activity is induced in a concentration-dependent manner at 5, 10, 15 and 20 mg per ml of EGCG. An 8-fold increase in activity is observed at 20 mg per ml. This response appears to be physiologic, as a parallel increase in endogenous hINV gene expression is observed. The activation is comparable to that observed for phorbol ester, a known keratinocyte differentiating agent. Mutation of a functionally important AP1 site, AP1-1, located within the hINV gene promoter proximal regulatory region (PRR), eliminates this regulation, suggesting that the response is mediated via an AP1 transcription factor-dependent mechanism. Fra-1, Fra-2, fosB, junB, junD, c-jun and c-fos levels are increased by EGCG treatment. Moreover, AP1 factor binding to the AP1-1 site is increased by EGCG and this complex contains Fra-1 and junD. Thus, our results indicate that EGCG markedly increases AP1 signaling, and differentiation-associated gene expression in normal keratinocytes. This is in sharp contrast to previous reports in transformed keratinocytes, and indicate that the mechanism of EGCG action is markedly different in normal vs. immortalized/transformed keratinocytes.

## 801

### Regulation of Human Involucrin Gene Expression by Serine/Threonine Protein Phosphatase Inhibitor Okadaic Acid

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Involucrin is a component of the keratinocyte cornified envelope that is an important marker of keratinocyte terminal differentiation. Okadaic acid (OA) is a potent and specific inhibitor of serine/threonine protein phosphatases PP2A and PP1. The role of okadaic acid-sensitive phosphatases in epidermal differentiation has not been elucidated. In the present study, we examine the effect of this agent on involucrin gene expression. We show that OA enhances human involucrin (hINV) promoter activity in cultured human epidermal keratinocytes, and potentiates phorbol ester-dependent activation of the promoter. The endogenous hINV protein level is also increased by OA treatment, suggesting that inhibition of OA-sensitive phosphatases induces keratinocyte differentiation. Regulation of hINV promoter activity by OA requires the AP1-1 and C/EBP binding sites within the hINV promoter proximal regulatory region (PRR), and activation of hINV gene expression is associated with an OA-dependent increase in both AP1 and C/EBP DNA binding activity, respectively, at the hINV promoter AP1-1 and C/EBP sites. In addition, OA increases the expression of the AP1 transcription family members c-Jun, JunB, JunD, Fra-1 and Fra-2, and alters their phosphorylation state. This increase does not require protein kinase c, as pretreatment with PKC-specific inhibitor, bis-indolylmaleimide, does not block the OA-associated increase in AP1 protein level. C/EBP $\alpha$  and C/EBP $\beta$  level are also increased, and their phosphorylation state is altered by OA in a time- and a concentration-dependent manner. These changes are associated with an OA-dependent increase in p38 and JNK activity. Thus, OA appears to increase hINV gene expression via a mechanism whereby the p38 and JNK MAPKs increase AP1 and C/EBP factor levels and DNA binding.

## 803

### Different Properties of Three Isoforms of Transcription Factor AP-2 in Human Keratinocytes

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Transcription factor AP-2/promoter system is essential for the complex keratinocyte biology mediated by inflammatory cytokines, as we demonstrated previously (JID 113:600-6, 1999). Three isoforms, AP-2 $\alpha$ ,  $\beta$ , and  $\gamma$ , share a high homologous structure, but their functions are considered to be different. We therefore studied the implication of each AP-2 isoform in the keratinocyte proliferation, differentiation, and carcinogenesis. Serial skin sections from different sources, normal, psoriasis, and squamous cell carcinoma (SCC), were examined for AP-2 immunoreactivity. AP-2 $\alpha$  was present only in the nuclei of normal basal keratinocytes, but significantly increased in the lesional keratinocytes of both diseases. AP-2 $\beta$  was completely absent in all samples, whereas AP-2 $\gamma$  was homogeneously observed throughout the epidermis in normal and psoriatic skin as well as the SCC lesion. There was a strong correlation in the expression pattern between AP-2 isoforms and major keratinocyte markers such as keratin K1, K14, transglutaminase (TGase) I, and epidermal growth factor receptor (EGFR). Furthermore, *in vitro* DNA binding assay revealed the isoform-specific gene activation. AP-2 $\alpha$  was highly accessible with the promoter fragments of K14 and EGFR rather than those of K1 and TGase I, all of which are critical regions for the AP-2-dependent gene activation. In contrast, AP-2 $\gamma$  bound to these four promoter fragments with similar affinities, but AP-2 $\beta$  did not despite the binding with AP2 consensus oligonucleotide. These results suggest that three AP-2 isoforms perform unique properties in the spatial and temporal expression of keratinocyte-related genes, thereby maintaining the epidermal homeostasis. Disruption of the epidermal AP-2 balance may lead to the establishment of hyperproliferative and neoplastic phenotypes, such as psoriasis and SCC.

## 800

### The Human Involucrin Gene Contains Spatially Distinct Regulatory Elements that Regulate Expression During Early Versus Late Epidermal Differentiation

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Human involucrin (hINV) is a keratinocyte protein that is expressed in the suprabasal compartment of the epidermis and other stratifying surface epithelia. Involucrin gene expression is initiated early in the differentiation process and is maintained until just prior to terminal cell death. The distal regulatory region (DRR) is a segment of the hINV promoter (nucleotides -2473/-1953) that accurately recapitulates the normal pattern of suprabasal (spinous and granular layer) expression in transgenic mouse epithelia. To identify sequences that mediate expression at specific stages of differentiation, we divided the DRR into two segments, a 376 nucleotide upstream region (DRR-2473/-2100) and a 147 nucleotide downstream region (DRR-2100/-1953), and evaluated the ability of these sequences to drive expression in transgenic mice. The DRR-2473/-2100 segment drives expression at a level comparable to that observed for the DRR, but expression is restricted to the upper granular layers (i.e. no spinous layer expression). In contrast, the DRR-2100/-1953 segment does not drive expression. However, reassembling the DRR restores the complete range of expression. These results suggest that two distinct, spatially separate elements are required to specify the complete differentiation-dependent program of involucrin gene expression. To identify specific transcription factor binding sites involved in this region, we mutated an activator protein-1 binding site, AP1-5, located within DRR-2473/-2100 segment. This site binds AP1 transcription factors present in mouse epidermal extracts, and its mutation eliminates appropriate hINV expression. This result suggests that a multiprotein complex that forms over multiple, spatially separate, regulatory elements is required for appropriate expression of the hINV gene, and that AP1 factors participate as components of this complex.

## 802

### AP-1 and Sp1 Cooperatively Regulate Differentiation-Specific Expression of the Mouse Lorincrin Gene Via Direct Association

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We have previously shown that an AP-1 element in the proximal promoter of the mouse lorincrin gene is necessary but not sufficient for expression *in vivo* in transgenic mice. To identify additional regulatory elements required for expression of the lorincrin gene, we performed deletion and mutation analysis of the promoter region. Deletion or mutation of a 8-bp sequence (GGGAGGAG), which is located 14bp upstream of the AP-1 element, resulted in a significant decrease in promoter activity to a level similar to that observed by disruption of the AP-1 element. Electrophoretic mobility shift assays and supershift assays with specific antibodies showed that Sp1 and Sp3 specifically bound to this sequence. Overexpression of Sp1 in cultured keratinocytes resulted in a 12-fold enhancement of the promoter activity, however, Sp3 did not increase promoter activity. Immunoblotting studies using nuclear and whole cell extracts from undifferentiated or differentiated keratinocytes indicated that Sp1 translocated to nucleus in differentiated keratinocytes, potentially inducing differentiation-specific expression of the lorincrin gene. Further support for this hypothesis was obtained by cotransfection experiments which demonstrated that c-fos and Sp1 synergistically activated lorincrin promoter activity. Interestingly, jun family members (c-jun, JunB, JunD) suppressed the positive effect of c-fos on the lorincrin promoter. Site-specific mutagenesis of either the AP-1 and/or the Sp1 element indicated that both elements were essential and acted synergistically for differentiation-specific expression of the lorincrin gene. These data suggested functional cooperation between AP-1 and Sp1, therefore we examined the physical interaction between these two factors. *In vitro* binding by means of a glutathione S-transferase pull down assay showed that both c-jun and c-fos could directly associate with both Sp1 and Sp3. Taken together, these data provide evidence for both physical and functional interactions between AP-1 and Sp1 during differentiation-specific expression of the lorincrin gene.

## 804

### Transcriptional Regulation of Human Papillomaviruses by Interferon Regulatory Factor-1

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Human papillomaviruses (HPVs) are frequently associated with various lesions of the squamous epithelium. Cell-mediated immune responses against HPV infection result in release of various cytokines that in turn could regulate HPV transcription. Interferon regulatory factor-1 (IRF-1) is induced by several cytokines, and it activates transcription of target genes by binding to a specific sequence in their promoter region. Here we describe the properties of an Interferon Responsive Element (HIRE) in close proximity to the TATA box of HPV type 16. This site resembles the consensus IRF-binding element (IRF-E) or interferon-stimulated response element (ISRE) and overlaps viral E2 binding sites. HPV16 HIRE-1 binds IRF-1 protein in an inducible manner. Mutational analyses revealed the importance of crucial nucleotides in this binding. In a reporter system we demonstrated that HIRE-1 stimulates transcription in response to IRF-1 from both homologous and heterologous promoters in a dose-dependent manner. We also analyzed HIREs of some known mucosal HPV types by gel-shift assay and showed that some high-risk, but not low-risk, mucosal types also bind IRF-1. We can assume that transcription of those HPV types is regulated via HIRE in a fashion similar to that of HPV16. These results might provide important insights regarding the immune responses against HPVs.